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We demonstrated in the past that chemical conjugation of polyethylene glycol (PEG) moieties to recombinant human acetylcholinesterase (rHuAChE) gives rise to OP bioscavenger species which reside for very long periods of time in the circulation of mice, regardless of their post-translation-modification state. These findings allow for production of rHuAChE not only in eukaryotic cells but also in bacterial cost-effective systems. A synthetic human AChE gene was constructed and its expression was analyzed under control of various potent transcription promoters in *Bacillus brevis* cells. The potential use of this system for large-scale production of AChE is presently being assessed.

We have begun a series of studies to determine the effect of removal of human AChE lysine residues, which serve as target sites for PEG-conjugation, on the enzymatic and pharmacokinetic performance and on the degree of homogeneity of the enzyme product. In other studies we demonstrated that AChE PEGylation results in a major reduction of the immunogenicity of the enzyme.

In structure-function studies of AChE, we compared the reactivities of enantiomers of VX and their noncharged isosters, as well as ecothiophate, in conjunction with a battery of AChE mutants. These studies allowed us to define two subsites, located in the active site and peripheral anionic subsites, which confer enzyme stereoselectivity to CW agents such as VX.

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CONTENTS

I. GENERAL INTRODUCTION

The primary role of acetylcholinesterase (acetylcholine acetylhydrolase 3.1.1.7, AChE) is the termination of impulse transmission in cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine (ACh). Some organophosphate (OP) compounds, such as the nerve agent sarin and soman, inhibit AChE irreversibly by rapid phosphonylation of the serine residue in the enzyme active site. The acute toxicity of these nerve agents is elicited in motor and respiratory failure following inhibition of AChE in the peripheral and central nervous system.

The high reactivity of ChEs towards OP-agents led to propose these biomolecules as exogenous scavengers for sequestration of toxic OP-agents before they reach their physiological target (Wolfe *et al.*, 1987; Raveh *et al.*, 1989; Broomfield *et al.*, 1991; Doctor *et al.*, 1992). Exploitation of the bioscavenging potential of the recombinant bioengineered mutant derivatives of AChE depends on large-scale production systems. However, pharmacokinetic studies (Kronman *et al.*, 1992; Mendelson *et al.*, 1998) have shown that recombinant enzymes generated by these systems, relying on either bacterial or mammalian cells, are retained in the circulation of experimental animals for much shorter periods of time than native fetal bovine serum AChE (FBS-AChE) or human serum butyrylcholinesterase (BChE). Extensive structural and biochemical analyses of over twenty forms of recombinant AChEs allowed us to determine an hierarchical pattern by which post-translation-related factors as well as specific amino-acid epitopes, determine the pharmacokinetic performance of the enzyme molecule in animal models (Kronman *et al.*, 2000; Chitlaru *et al.*, 2001, 2002; Cohen *et al.*, 2004). Optimization of some parameters affecting circulatory residence of rHuAChE through cellular and DNA engineering allowed us to generate a circulatory long-live recombinant HuAChE. In parallel, we examined the possibility to convert the rapidly cleared rHuAChE into circulatory long-living enzyme species, by chemical modification. Controlled conjugation of polyethylene-glycol (PEG) side-chains to lysine residues of rHuAChE resulted in the generation of active enzyme demonstrating unprecedented pharmacokinetic performance (Cohen *et al.*, 2001, 2004).

To define the possible interrelationship between PEG-conjugation and post-translation modifications of AChE enzyme forms with regard to circulatory residence, we examined the pharmacokinetic performance of well-defined AChE forms differing in their post-translation processing. This line of studies allowed us to determine that PEG-conjugation increased the circulatory retention of suboptimally processed AChE forms characterized by low glycan contents, inefficient sialylation or incomplete assembly into tetramers. Moreover, AChE species exhibiting altered glycan structures that do not conform with the classical complex-type of oligosaccharides typical of animal cell proteins or which were entirely devoid of glycan appendages, also displayed prolonged circulatory retention following PEG-conjugation. Most notably, the differently processed AChE forms displayed nearly equal circulatory residence time values subsequent to PEGylation. Taken together, these studies show that PEGylation of AChE overrides post-translation-related suboptimal processing and that the extent of circulatory retention of the chemically modified enzyme is determined solely by the appended PEG moieties.

A key implication of these conclusions is that it is now possible to consider production of recombinant human AChE in low-cost microorganisms-based production systems. To this end, we generated a synthetic *ache* gene which has been adapted for efficient expression in microorganisms by lowering the GC contents of the coding sequences of the native human gene, and demonstrated that this synthetic gene expresses authentic AChE *in vitro*. Expression of this gene in various microorganism expression systems of bacterial or yeast origin, will allow us to evaluate which of these systems may serve in the future for the large-scale cost-effective production of this OP-bioscavenger. The present report summarizes the studies carried out throughout the last year regarding expression the synthetic AChE gene in bacterial cells.

Although PEG-conjugation of AChE in itself leads to the generation of a circulatory long-lived species, the practical use of PEGylated AChE as an OP-bioscavenger would require that the enzyme product exhibit a high degree of homogeneity. This may demand the removal of some of the lysine residues which serve as targets for PEGylation, to allow production of uniformly PEGylated enzyme. To this end, we began a series of studies aimed to determine the effect of lysine removal on enzyme functionality, pharmacokinetic performance and structural homogeneity following PEGylation. In parallel, we began a set of experiments designed to determine the effect of PEG conjugation on the immunological characteristics of wild-type and lysine mutated AChEs. In the present report we document our findings to date with regard to all these issues.

Finally, in the present study we summarize our findings regarding the stereoselectivity of HuAChE toward the enantiomers of VX, through comparisons with their noncharged isosteres, as well as with the symmetrical diethyl phosphates echothiophate and its noncharged analogue. These agents were analyzed with a battery of HuAChEs mutated at the different binding subsites of the enzyme active center gorge. Our results indicate that in the case of VX, stereoselectivity is a result of multiple interactions involving several elements of the active center and the peripheral anionic site, including polar interactions with the charged leaving group.

II. Generation of rHuAChE in Microorganism-Based Expression Systems

INTRODUCTION

As we have documented at length (Midterm report , contract DAMD-17-03-C-0012, March 2004), optimized PEG-conjugation results in the long-term retention of AChEs in the circulation even when these are not optimally processed. Thus, proteins lacking glycan moieties or containing glycans which are not capped by sialic acid, exhibit extended circulatory retention following PEG-conjugation, probably due to the masking effect of the appended PEG which obstructs removal via receptor-dependent elimination systems. Likewise, assembly of AChE into tetramers is not a prerequisite for circulatory longevity, most likely since the molecular size of the PEGylated enzyme decreases or prevents clearance through glomerular filtration. This body of data point towards the potential ability to utilize recombinant human AChE produced in non-mammalian production systems, as a cost-effective source for large-scale amounts of enzyme which can then be efficiently PEGylated to generate circulatory long-lived enzyme species for therapeutic use.

Generation of human acetylcholinesterase in an *E. coli*-based production system was documented in the past (Fischer *et al.*, 1993). However, the recombinant enzyme produced in this system segregated as misfolded protein forms in insoluble inclusion bodies, and only 3% of the recombinant AChE could be successfully refolded into enzymatically active forms.

Bacillus species synthesize and secrete many extracellular enzymes directly into the medium in high yields (Simonen and Palva, 1993) and therefore may serve as an attractive alternative to *E. coli*, for expression and secretion of properly folded recombinant proteins. Indeed, high level production and extracellular secretion of recombinant enzymes were achieved in *Bacillus*-based systems using different combinations of parameters which affect production. In most cases the host strain were either the six or seven protease-deficient strains of *B. subtilis* (WB600, WB700, W751) or low protease strains of *B. brevis* (HPD31, *B. brevis* 47). In some cases, the signal peptide of the protein of interest was modified to comply with the general structure of *Bacillus* signal peptides, but in most cases the original signal peptide was replaced with a *B. subtilis* or *B. brevis* signal. Likewise, the promoter driving transcription was usually of *Bacillus* origin (e.g. Pamy of *B. amyloliquefaciens,* P43 of *B. subtilis*, cell wall protein promoter of *B. brevis*). Extensive studies designed to find an optimal combination of host cells (*B. subtilis* vs. *B. brevis*, examination of specific strains), signal-peptide, signal-protein joining procedure, and transcription promoter, led to high-level production and secretion of recombinant proteins within the 200-1000 mg/liter range.

In view of this body of information, we investigated the possibility to utilize a *Bacillus*-based expression system for the production of recombinant human AChE at large-scale, as detailed below.

RESULTS

As reported previously (Midterm report , contract DAMD-17-03-C-0012, March 2004), we designed and a constructed a synthetic HuAChE gene (sAChE) of lower GC content, which codes for authentic human AChE, utilizing nucleotide codons which will be compatible with efficient expression in microorganisms-based systems. The synthetic human AChE coding region sequences, including a *Bacillus*-oriented ribosome binding site and the *B. brevis* cell wall protein signal peptide (Tsuboi, *et al.*, 1986; Yamagata *et al.*, 1987), were assembled in tandem to generate the full-length coding region of the human AChE gene and cloned into the pGEM3 vector. This AChE-containing pGEM3-based vector, which contains the T7 promoter upstream to the sAChE sequence, was subjected to radioactively labeled *in-vitro* transcription-translation, followed by immunoprecipitation with polyclonal mouse anti-HuAChE antibodies. A single major protein product corresponding to full-length AChE was specifically precipitated by the anti-AChE antibodies, verifying that genuine human AChE enzyme was indeed generated.

To allow expression of the synthetic AChE in *Bacillus* systems, the synthetic AChE coding region was cloned into the *E. coli-Bacillus* shuttle vector pXX_{MCS-5} (Gat *et al.*, 2003) downstream to three different promoters: (1) the α-amylase promoter of *Bacillus amyloliquefaciens*, P*amy*, (2) the *Bacillus anthracis* surface antigen protein promoter, $Psap_{long}$ and (3) the *Bacillus brevis* cell wall protein promoter, P*cwp*. The P*amy* promoter has been shown in the past to drive high levels of expression of recombinant protein in *B. subtilis* and *B. anthracis* (over 100 mg/ml, Cohen *et al.*, 2000). The P*sap* promoter controls expression of the S-layer proteins, which are often the most abundant proteins in bacterial cells, and is considered to be among the strongest known in nature. Indeed expression of heterologous genes under control of the P*sap* promoter, resulted in an 8-fold increase in production levels as compared to P*amy* promoter-driven expression of the same genes (Gat *et al.*, 2003). The P*cwp* promoter controls expression of the cell wall proteins in *B. brevis* (Adachi *et al.*, 1991), and can be regarded as the *B. brevis* counterpart to the *B. anthracis* P*sap* promoter.

The synthetic HuAChE gene, together with the *B. brevis* cell-wall protein ribosomal binding site (RBS) and signal peptide (SP) were excised from the pGEM3-sAChE vector, and used to replace the GFP coding region and RBS in the $pGuv_{amy}$ and $pGuv_{sapL}$ vectors (Gat *et al.*, 2003). The resulting constructs, $psAChE_{amy}$ and $psAChE_{sapL}$, contain the sAChE coding sequence under control of P*amy* and P*sap* promoters, respectively (Figure 1). For the generation of P*cwp*-controlled sAChE, P*cwp* specific primers were utilized for PCR amplification of the P*cwp* region (Adachi *et al.*, 1991), using *B. brevis* chromosomal DNA as template. The resulting PCR product was verified by sequencing, and was used to replace the Pamy promoter of $psAChE_{amy}$ to generate $psAChE_{cwp}$ (Figure 1).

For expression of the synthetic rHuAChE gene, we chose the *B. brevis choshinensis* strain (Takagi *et al.*, 1993), which has been shown in the past to sustain high levels of expression and secretion of recombinant proteins from various sources (e.g. Ebisu *et al.*, 1996; Nagahama *et al.*, 1996; Takimura

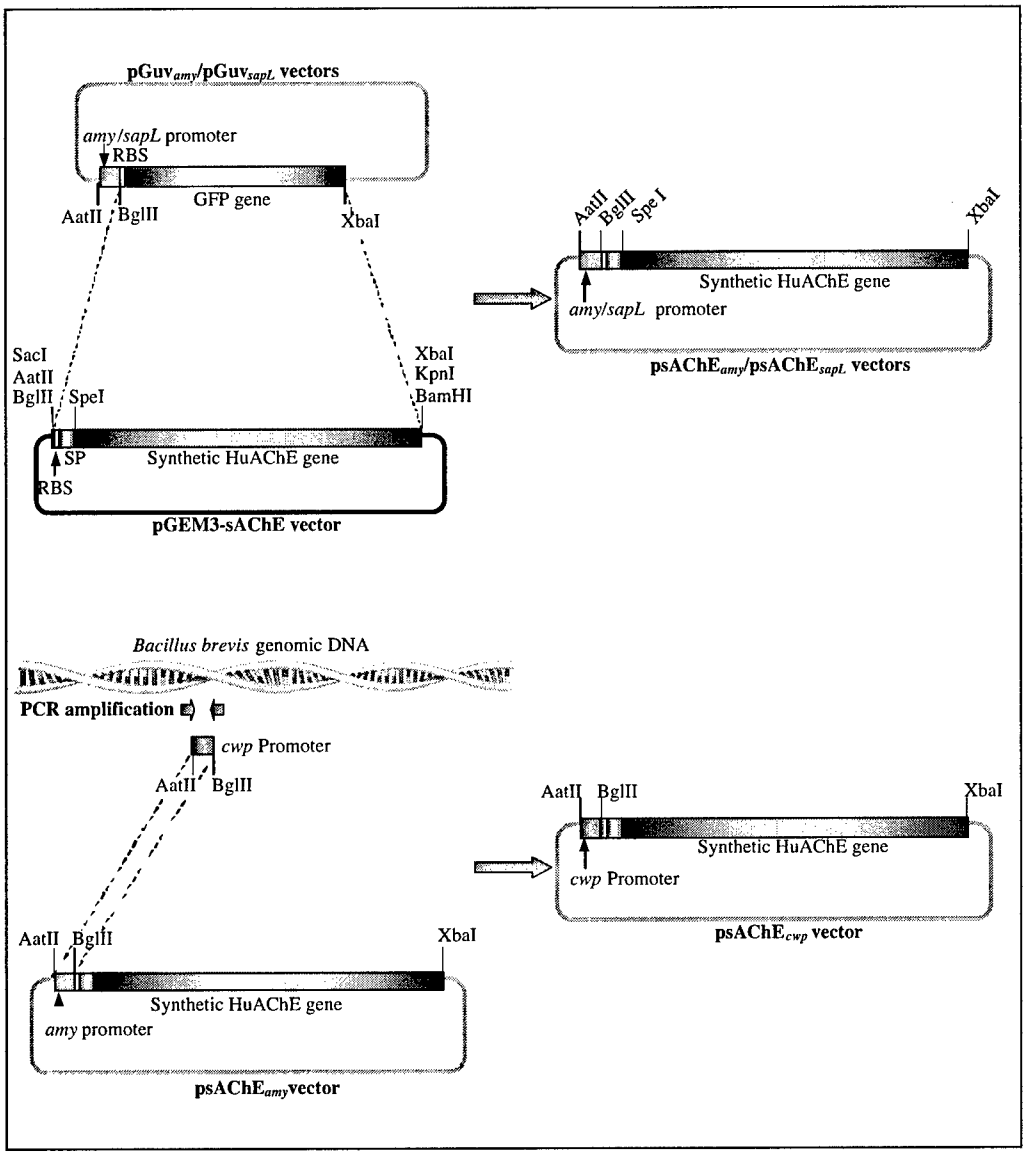


Figure 1: Description of the construction of the three *Bacillus*-oriented synthetic AChE expression vectors. SP - Signal Peptide, RBS - Ribosomal Binding Site, GFP - Green Fluorescent Protein,

et al., 1997). Competent cells were prepared by a calcium chloride method adapted for these cells (Okamoto *et al.*, 1997), and plasmid DNA was introduced by electroporation, following meticulous calibration of the settings for optimal cell transformation. The three *Bacillus*-oriented sAChE expression vectors were introduced into *B. brevis* cells, which were then selected for neomycin resistance (Figure 2). Several neomycin resistant clones were examined for the presence of the expression plasmid by PCR analysis, utilizing internal primers for the synthetic AChE gene. In all cases, the transformed clones were shown to contain the AChE gene.

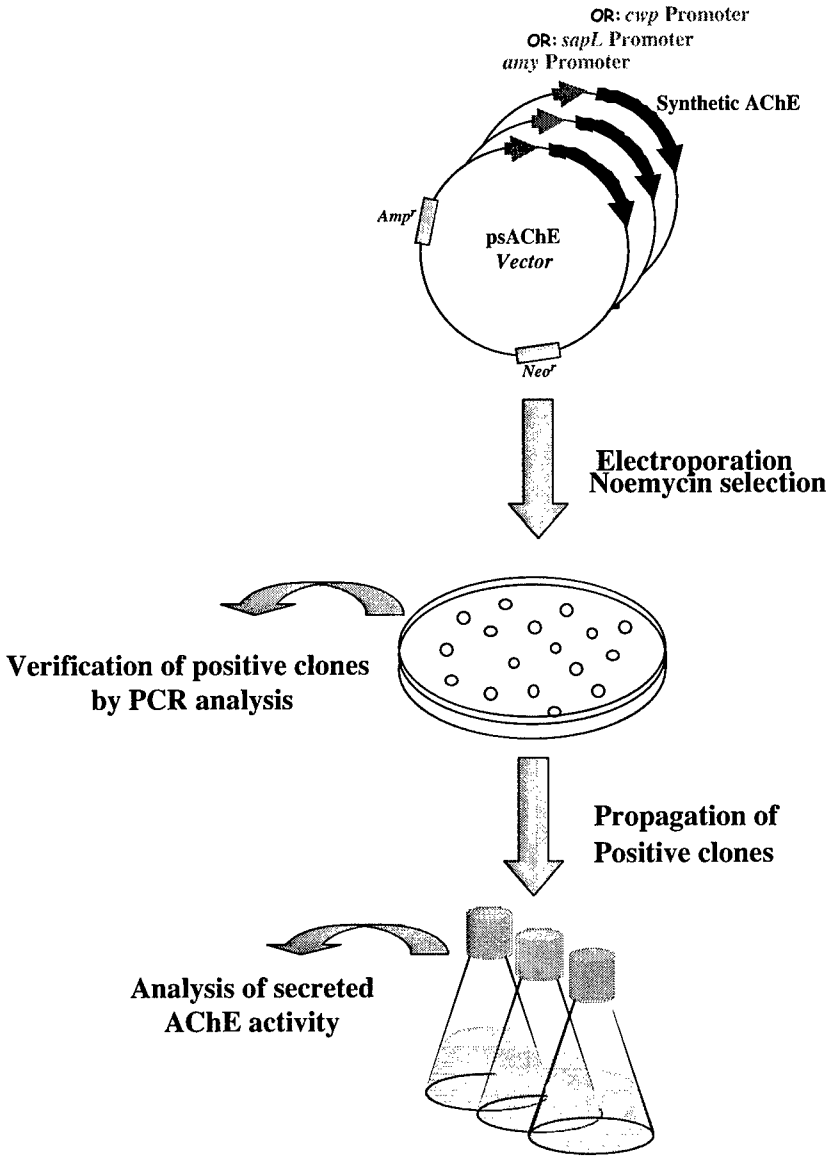


Figure 2: Schematic presentation of the generation and initial analyses of human AChE transformed *B. brevis* cells.

Careful inspection of cell clones expressing each of the different constructs failed to detect AChE activity in the growth medium at both the logarithmic and stationary phase. We therefore examined whether AChE activity can be detected within the bacterial cell. Indeed, Western immunoblotting analysis (Figure 3) allowed us to determine the presence of intracellular AChE at logarithmic phase (8 hours culture), and to a lower extent at stationary phase (16 hours). However,

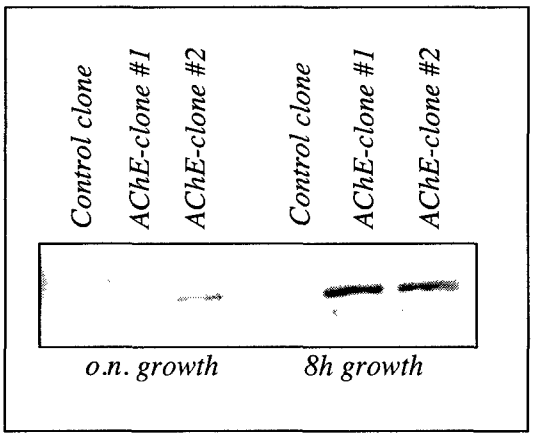


Figure 3: Western blot analysis of intracellular recombinant human AChE in *B. brevis* cells. *B. brevis* cells stably transformed with the psAChE$_{amy}$ AChE expression vector or control vector were propagated . Samples (1 ml) were removed at 8 hours and 16 hours, intracellular protein was extracted, and then subjected to Western blot analysis using anti-HuAChE polyclonal antibodies (Shafferman *et al.*, 1992). Similar results were obtained for cells transformed with the psAChE$_{sapL}$, expression vectors (not shown).

densitometric analysis of intracellular AChE revealed that the quantities of enzyme are very low, 1 μg/liter cell culture. This unexpectedly low level of enzyme expression (as compared to GFP production), may stem from an inefficiency of regulatory elements within the expression vector. To address this issue, we constructed control plasmids in which the AChE coding region was substituted by that of the green fluorescence protein (GFP) gene. *B. brevis choshinensis* cells were transformed with the pGuv$_{amy}$ expression vector, and several neomycin resistant clones were examined for GFP expression. High level GFP expression was evident by UV illumination (Figure 4A). Indeed, Western blot analysis followed by densitometric quantitation of the intracellular GFP (Figure 4B), allowed us to determine that the expressed protein reached a level 10,000-fold higher than AChE (10 mg/liter cell culture). Thus, the expression vehicles used in these studies, are fully capable of supporting high-level expression of heterologous proteins in the *B. brevis choshinensis* cells. The low level of AChE gene product in the bacterial cells, may therefore be an outcome of low transcription/translation levels, or conversely, from an instability of either the message RNA or the protein product.

To determine whether the low level of AChE gene product in the bacterial cells is due to low transcription levels or to an instability of the sHuAChE message RNA, transformed cells (10^9 cells) expressing heterologous rHuAChE or rGFP were harvested at log-phase growth, and total RNA was isolated and subjected to Northern blot analysis (Figure 5). Detection of the rHuAChE and rGFP mRNAs was carried out with equal amounts of ^{33}P-ATP labeled 100-bp probes complementary to the 3'-end of the corresponding transcripts. Following fluorography, the ~1850 base-long band

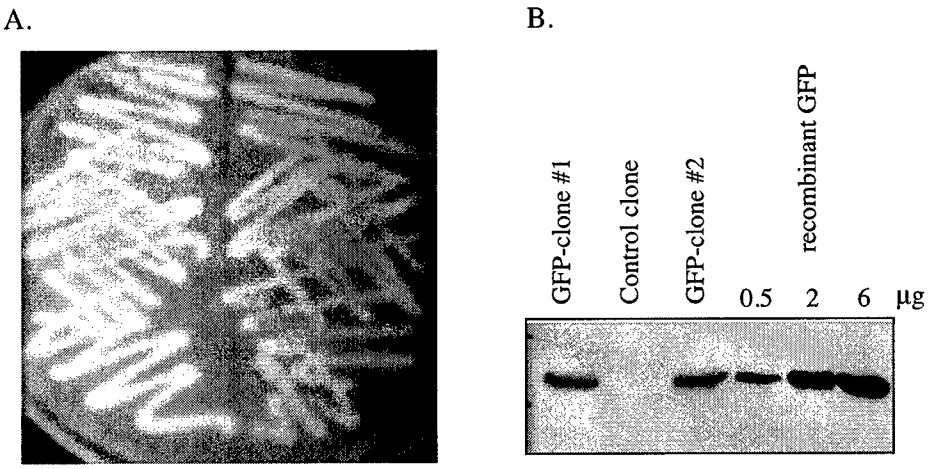


Figure 4: Expression of GFP in *B. brevis* cells. **(A)** *B. brevis* cells stably transformed with the pGuv$_{amy}$ GFP expression vector were streaked unto LB agar plates and inspected by UV light (366 nm). **(B)** *B. brevis* cells stably transformed with the pGuv$_{amy}$ GFP expression vector or control vector were propagated . Samples (1 ml) were removed, intracellular protein was extracted, and then subjected to Western blot analysis using anti-GFP polyclonal antibodies (Clontech Inc.). Recombinant GFP (Upstate biotechnology Inc.) loaded on the same gel at different quantities served as a standard curve for densitometric quantitation. Similar results were obtained for cells transformed with the pGuv$_{sapL}$ expression vectors (not shown).

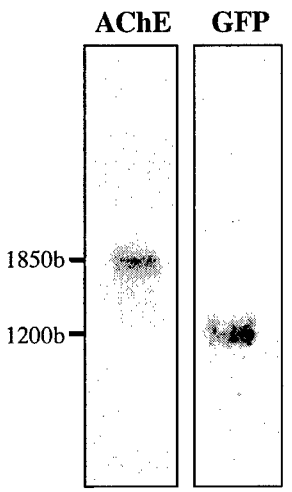


Figure 5: Northern blot analyses of rAChE and rGFP mRNAs expressed in transformed *B. brevis* cells. Equal amounts of total RNA isolated from *B. brevis* cells transformed with the *E. coli-Bacillus* shuttle vector pXX$_{MCS-5}$ carrying the rHuAChE gene (left) or rGFP gene (right) were denatured with glyoxal/DMSO, resolved on agarose gels and subjected to Northern blot analyses. Detection of the respective mRNAs was achieved by hybridization to ^{33}P-ATP labeled 100-bp probes complementary to the 3'-end of the corresponding transcripts, followed by fluorography.

corresponding to the AChE transcript and the ~1200 base-long band corresponding to the GFP transcript displayed very similar intensities, attesting to the nearly equal amounts of the two heterologous RNAs within the respective cells at steady-state. The finding that the intracellular level of rGFP protein is 10,000-fold higher than that of intracellular rHuAChE protein, even though transcript levels of the corresponding RNAs are similar, suggests that the limitation in rHuAChE production is a result of either inefficient translation or instability of the translated protein product.

In summary, a synthetic human AChE gene adapted for expression in bacterial systems, was expressed in *B. brevis* cells under control of various potent transcription promoters of *Bacillus* origin. Transformed cells containing the recombinant gene support high levels of AChE transcription yet, at present, these exhibit only low levels of the rHuAChE protein product. We are currently carrying out a series of experiments to resolve this issue and to determine, if and how, one can take full advantage of the *B. brevis* expression system for production of human AChE.

III. Generation of hypolysine mutants of rHuAChE as substrates for PEGylation

INTRODUCTION

The conversion of AChE into PEG-modified long-lived molecules which can serve as effective therapeutic bioscavengers requires that the chemically modified product: **(i)** reside in the circulation for sufficiently long-periods of time, **(ii)** retain its biological activity and, **(iii)** exhibit maximal homogeneity. The amount of appended PEG moieties may crucially affect each of the three requirements mentioned above: while increasing the PEG load of AChE may positively contribute to long-term circulatory residence, the enhanced pharmacokinetic performance of the enzyme may be associated with a concomitant loss in biological activity and/or may display increased heterogeneity.

Previous studies carried out in our laboratory demonstrated that under various sets of conditions, different amounts of PEG moieties can be appended to rHuAChE. This study led to the generation of an array of PEGylated AChEs which differed one from another by their degree of modification. Subjecting the differently PEGylated AChEs to pharmacokinetic studies allowed us to determine that the circulatory residence time values of the various enzyme forms is linearly correlated with the number of appended PEGs. PEGylated AChE containing an average of 4 to 5 PEG moieties per enzyme, displayed maximal pharmacokinetic performance, while retaining full catalytic activity (Cohen *et al.*, 2001). However, examination of the enzyme product revealed that it comprised a mixture of 3 major products, differing one from another in their PEG contents. Further attempts to increase the number of appended PEG units or to achieve uniform PEGylation under stringent conditions which favor highly effective PEG appendage, resulted in the generation of enzyme forms displaying severely reduced catalytic activity.

The generation of PEGylated AChE arrays displaying different characteristics in terms of pharmacokinetics, enzymatic activity and homogeneity may be achieved not only by altering reaction conditions, but also by reducing or increasing the number of lysine target residues in AChE. High levels of PEGylation may be achieved even under mild reaction conditions, if lysine target residues are added to the enzyme. On the other hand, the elimination of some lysine residues to prevent their appendage to PEG may be required to generate uniformly modified enzyme forms which retains full catalytic activity following PEGylation under stringent conditions which would otherwise reduce catalytic activity. For instance, PEGylation of the lysine at position 348 of human AChE, which is located at the entrance to the gorge leading to the catalytic site of the enzyme, may prove to sterically hinder the diffusion of substrate to the active center, and therefore should be eliminated. However, elimination of selected lysine residues should be restricted to those which are not required for catalytic activity, nor play a role in the reactivity of the enzyme towards organophosphates. One should also keep in mind that one more of the lysine residues may be crucial for maintaining the native enzyme architecture, as has been proven in the past for selected residues (Shafferman *et al.*, 1992). For example, localization of the lysine residues within the 3D model of rHuAChE demonstrates that some of these lysines are located within well-defined conformational structures such as α-helices, raising the question whether their replacement might negatively influence the conformational integrity of the enzyme. These various considerations suggest that a wide spectrum of PEGylated lysine-modulated

AChE forms be examined empirically to determine the optimal AChE configuration in terms of biological activity and pharmacokinetic performance. Such studies should include examination of the relationship between the number of available lysines and the pharmacokinetic performance of the PEGylated enzyme product.

Another point of consideration should take into account that the various lysines display an unequal distribution at the enzyme surface. Lysine elimination should therefore be designed not only in terms of the number of sites remaining for PEG appendage, but also should take into account the spatial location of the various lysine moieties, to avoid the generation of enzyme-surface exposed regions that are not protected by nearby PEGylated lysine residues.

RESULTS

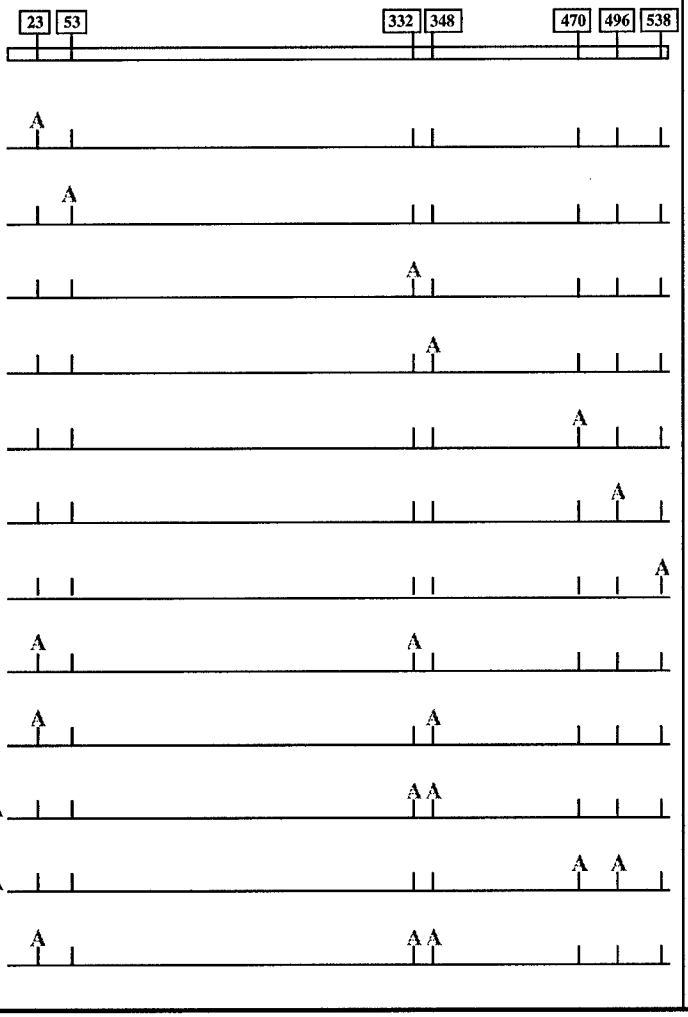
We have reported in the past (Midterm report, contract DAMD-17-03-C-0012, March 2004) that following PEGylation, wild type rHuAChE, monomeric C580A rHuAChE and the C-terminal truncated version of rHuAChE (ΔC- rHuAChE) displayed nearly identical pharmacokinetic profiles, characterized by very similar half-life and mean residence time values. These findings demonstrated that the variance in number of accessible lysines in the these enzyme preparations (10 for WT and C580A rHuAChE; 7 for the ΔC- rHuAChEs) did not affect the ability of the enzyme to undergo efficient PEGylation. We noted however that this apparent insensitivity to lysine number may simply reflect the fact that in both of the enzyme species under examination, the lysine contents exceeds the maximal amount required for optimal circulatory retention, and that enzyme species characterized by lower lysine contents may display differential circulatory longevity following PEGylation.

As a first step towards the evaluation of the effect of low lysine contents on AChE pharmacokinetics, we generated by site-directed mutagenesis a series of different hypolysine AChE expression vectors. Each of the seven constructs (K23A, K53A, K332A, K348A, K470A, K496A, K538A), code for C-terminal truncated AChE in which a single lysine residue is replaced by alanine. These constructs were introduced into HEK-293 cells, and stable pools of each of these seven AChE mutants were generated. Protein quantities of the various hypolysine AChEs were determined by ELISA and their specific activities were calculated. In all cases, the specific activity of the hypolysine mutants did not deviate in a significant manner for that of the wild type enzyme (6.5 U/μg, see Table 1); at most, a ~3-fold decrease or increase in specific activities as compared to the wild type enzyme were observed. Taken together, these results indicate that the removal of any of the individual lysine residues does not alter the kinetic performance of the enzyme. Based solely on this criterion, any of the lysine residues may be eliminated to generate modified AChE for PEGylation, yet pertinent differences in the functional roles of the different lysine residues that were not detected by determining their specific activities, may yet be revealed by subjecting the various hypolysine forms to other examinations. To this end, the various hypolysine mutants were examined for thermal stability, to determine whether some of the lysines play a greater role in maintenance of the functional integrity of the enzyme, and thereby should not be replaced. This was achieved by incubating the monolysine mutated AChEs at 51^0C for various periods of time followed by measurement of residual enzymatic activity. The thermal decay curve of each of the mutant AChE forms was profiled and half-life time values were determined. Nearly all of

the hypolysine forms displayed thermal decay values, which did not differ significantly from that of the wild-type enzyme ($T_{1/2}51^0C$ = 8.9 min.). Only one of the hypolysine AChE forms (K470A) exhibited a greater than 5-fold reduction in thermostability as compared to the wild-type enzyme (Table 1).

Table 1: Specific activity and thermostability values of WT and hypolysine ΔC-AChEs[1].

AChE type	Specific activity (U/μg)	$T_{1/2}$ at 51°C (min.)
WTΔC-AChE (548aa)	6.5	8.9
ΔC-K23A	12.0	10.2
ΔC-K53A	4.1	8.4
ΔC-K332A	4.5	3.1
ΔC-K348A	2.2	4.6
ΔC-K470A	6.3	1.5
ΔC-K496A	20.0	8.9
ΔC-K538A	5.7	4.6
ΔC-K23A/K332A	10.8	3.3
ΔC-K23A/K348A	4.0	4.9
ΔC-K332A/K348A	2.5	3.0
ΔC-K470A/K496A	12.7	1.3
ΔC-K23A/K332A /K348A	2.5	2.6



1- framed numbers (upper line) designate positions of lysine residues in wild-type ΔC-AChE.

The finding that replacement of single lysine residues did not significantly alter the catalytic performance or thermostability of the enzyme, suggests that any of the various lysines may be replaced to generate hypolysine enzyme for controlled PEGylation. Preparation of homogenous PEGylated AChE, however, requires the generation of enzyme forms carrying multiple lysine replacements. As an intermediate step towards the generation of an array of such multilysine mutant AChEs, we generated several AChE forms, in which two lysine residues were replaced by alanine. These were introduced into HEK-293 cells and enzyme secreted from stable cells was characterized (Table 1). As in the case

of the single lysine replacements, the specific activities of the various double lysine mutants did not deviate significantly from that of the wild-type enzyme; at most, a 2.5-fold an increase or decrease in specific activities as compared to of the wild-type enzyme was observed. Examination of the various double lysine mutants for thermal stability allowed us to determine the thermal decay values of the various hypolysine mutants. Only enzyme forms which contained the K470A mutation exhibited a greater than 5-fold reduction in thermostability as compared to the wild-type enzyme. Based on these findings we decided to generate different multilysine AChE mutants in which three lysine residues are replaced by alanine. As a first step, the mutant K23A/K332A/K348A AChE gene was constructed. This multilysine AChE mutant (containing 4 lysine residues at positions 53, 470, 496 and 538) was introduced into HEK-293 cells, produced at-large-scale and purified. Measurement of the AChE activity of this multilysine enzyme mutant allowed us to determine that its specific activity did not deviate in a significant manner for that of the wild type enzyme. Likewise, examination of the K23A/K332A/K348A multilysine mutant for thermal stability allowed us to determine that its thermal decay compared to that of the wild-type enzyme.

Some of these hypolysine AChE enzyme forms will now be monitored for their pharmacokinetic behavior and immunological properties before and after PEG-conjugation. The pharmacokinetic and immunological profiles of these hypolysine mutants together with the assessment of their degree of uniformity following PEGylation, will serve as a guideline for further modification of the human AChE template for the generation of the optimally tailored enzyme forms.

IV. Determination of the Antigenic and Immunogenic Properties of PEGylated rHuAChEs

INTRODUCTION:

The use of mutagenized forms of human AChE and the possible requirement, in certain situations, for repeated administration of the prophylactic agent, are a cause for concern regarding the possible development of unfavorable immunogenic responses to the bioscavenger. PEG-modified therapeutic proteins have been documented to display decreased antigenicity and immunogenicity, as compared to their corresponding nonmodified parent molecule (Abuchowski *et al.*, 1977a,b; Koide and Kobayashi, 1983; Goodson and Katre, 1990; Katre, 1990; Chinol *et al.*, 1998). The attachment of a linear, flexible uncharged hydrophilic polymer to available but nonessential groups on an enzyme provides a shell around the enzyme that covers antigenic determinants and, by presenting a flexible hydrophilic surface for inspection by the immune processes, prevent recognition of the interior enzyme as a foreign substance against which an immune response would be provoked. Reduced degradation by antigen-presenting cells such as macrophages, shielding of some epitopes of peptides after degradation, or prevention of binding to receptors on B-cells, may all play a role in conferring reduced immunogenicity to the PEG-modified protein (Tsusumi *et al.*, 2000).

The amount and specific locations of the appended PEG molecules on the protein surface may be of importance in determining the immunogenicity of modified proteins. The array of PEGylated-AChE enzymes in which one or more lysine target sites were removed, can serve to examine the contribution of different amounts of PEG or their specific locations at the enzyme surface to the immunogenic and antigenic properties of the PEGylated AChE product. Determination of the antigenic potential of different surface regions of the enzyme will allow judicious selection of specific configurations of lysine residues for PEGylation, on the basis of their ability to support the generation of modified enzyme displaying both homogeneity and low immunogenicity.

RESULTS:

As an initial step towards determination of the immunological properties of PEGylated rHuAChE, antigenicity studies were carried out with nonmodified ΔC-rHuAChE and PEGylated rHuAChE. To this end, 160 ng of either ΔC-rHuAChE or PEGylated rHuAChE were incubated with different amounts of polyclonal mouse anti-HuAChE (Shafferman et al., 1992), and then precipitated with Protein G sepharose beads. Immunoprecipitation was evaluated by determining residual AChE activity in the supernatant. The results (Figure 6) clearly demonstrate that, unlike the nonmodified enzyme, the PEGylated version of the enzyme is ineffectively precipitated by the anti-AChE antibodies, suggesting that the appendage of PEG moieties efficiently "shield" the enzyme from immuno-recognition.

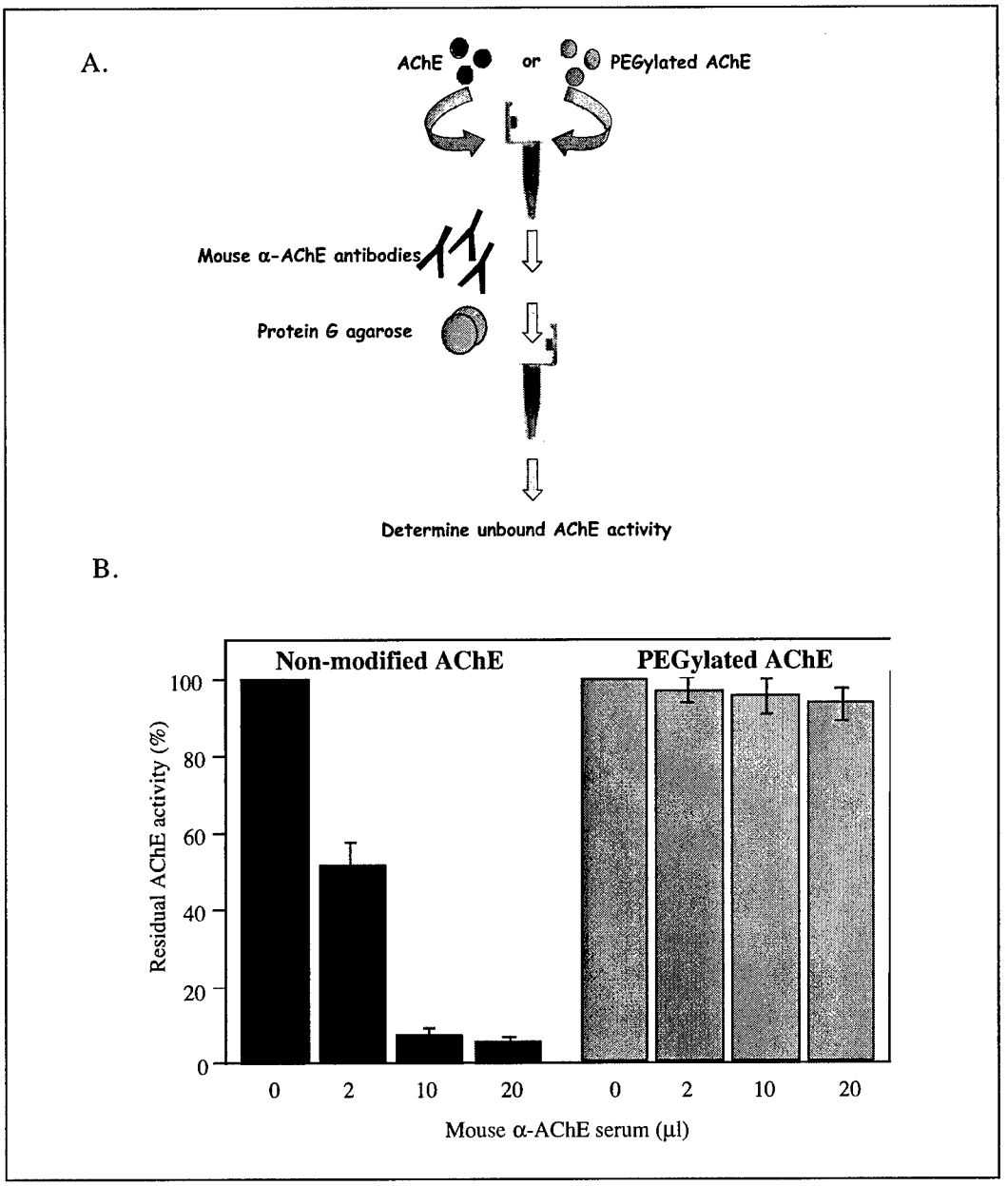


Figure 6: Determination of antigenicity of AChE and PEGylated AChE. A. Presentation of the immunoprecipitation scheme: AChE or PEGylated AChE proteins were incubated in the presence of different amounts of mouse polyclonal anti-AChE antiserum and then precipitated with Protein G-agarose beads. Residual AChE activity in the soluble nonbound fraction was determined. B. Residual AChE activity following immunoprecipitation with the indicated amounts of antiserum.

To further evaluate the effect of PEGylation on the immunological properties of AChE, we compared the immunogenic potential of PEGylated rHuAChE to that of nonmodified rHuAChE, following their administration to mice. To this end, non-modified or PEG-modified AChE (no adjuvant included) were repeatedly administered to mice (n=10) at monthly intervals, and anti-AChE antibody formation was monitored (Figure 7). Mice administered with non-modified AChE displayed substantial

levels of anti-AChE antibody even after the second administration (antibody titers = 1500 at 7 weeks), and antibody levels increased significantly following the administration of a third dose (antibody titers = 7800 at 11 weeks). In contrast, anti-ChE levels were consistently low throughout the experiment (antibody titers were less than 400 at 11 weeks). The failure of PEG-AChE to elicit significant antibody formation even in a heterologous animal model (human AChE in mice), strongly suggests that the PEG appendage results in the conversion of the AChE molecule into an "immunologically inert" form. Thus, PEG-appendage may contribute to the therapeutic potential of AChE, not only by improving the pharmacokinetic properties of the enzyme, but also by effectively shielding the enzyme molecules from the host immune system.

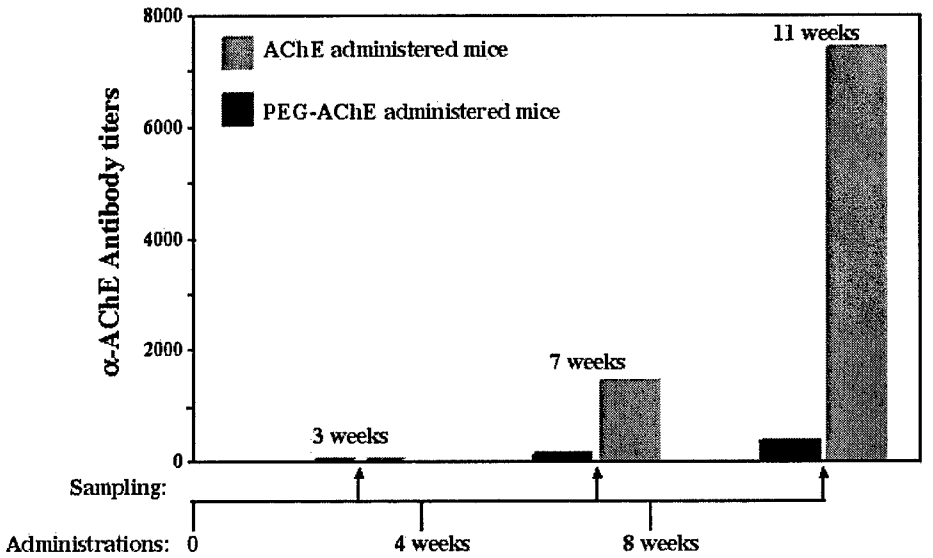


Figure 7: Anti HuAChE antibody titers in mice administered with rHuAChE or PEGylated rHuAChE. Mice (10 mice/group) were administered at monthly intervals with 10 μg/mouse of either rHuAChE or PEGylated rHuAChE without adjuvant. Antibody titers were determined in blood samples removed at various periods of time, as indicated in the figure. Antibody titers were determined by direct ELISA, in 96-well microtiter plates coated with highly purified rHuAChE.

Taken together, these experiments demonstrate that the appendage of PEG moieties to HuAChE effectively prevents enzyme accessibility to the host immune system. However, one should bear in mind, that these experiments were carried out using the PEGylated wild-type enzyme. We therefore intend, in the future, to extend these studies to include examination of the immunological properties of selected hypolysine AChE mutants enzyme as well.

V. Stereoselectivity Toward VX is Determined by Interactions with Residues of the Acyl Pocket and the Peripheral Anionic Site of AChE

INTRODUCTION:

The catalytic efficiency of acetylcholinesterase and its high reactivity toward both covalent and noncovalent inhibitors are believed to originate from the unique architecture of the AChE active center (Kaplan *et al.*, 2001, 2004; Barak *et al.*, 2002;). Elements of this architecture, unraveled during over a decade by x-ray crystallography (Sussman *et al.*, 1991; Bourne *et al.*, 1995; Kryger *et al.*, 2000; Harel *et al.*, 2000), site directed mutagenesis and kinetic studies of the AChE mutants (Shafferman *et al.*, 1992; Radic *et al.*, 1992, 1993; Vellom *et al.*, 1993; Ordentlich *et al.*, 1993, 1998; Barak *et al.*, 1994; Ariel *et al.*, 1998), include a) the esteratic site containing the active site serine; b) the "oxyanion hole" consisting of residues Gly120(118*), Gly121(119) and Ala204(201); c) the "anionic subsite" or the choline binding subsite -Trp86(84) ; d) the hydrophobic site for the alkoxy leaving group of the substrate containing an "aromatic patch" that includes residues Trp86(84), Tyr337(330) and Phe338(331); e) the acyl pocket - Phe295(288) and Phe297(290) and f) the peripheral anionic site (PAS) localized at or near the rim of the active site gorge and consisting of residues Asp74(72), Tyr72(70), Tyr124(121), Trp286(279), Tyr341(334). Among the AChE ligands which have been used to probe the molecular environment of the active center, organophosphorus inhibitors such as dialkyl phosphates and methylphosphonates, are particularly suitable. Their unusually high reactivity toward the enzyme indicates efficient accommodation by the active center binding elements (Hosea *et al.*, 1995, 1996; Ordentlich *et al.*, 1996, 1999; Barak *et al.*, 2000). The high affinity toward OP inhibitors may result from their ground state tetrahedral geometry that mimics to some degree the spatial disposition of the intermediate ACh-AChE adduct (Hosea *et al.*, 1995; Ordentlich *et al.*, 1996). In the noncovalent OP-AChE complex, accommodation of the tetrahedral phosphoryl moiety includes polar interactions of the phosphyl oxygen with the oxyanion hole as well as that of His447 with the phosphate (or phosphonate) alkoxy oxygen. The alkyl moieties of the inhibitor are contained within the hydrophobic domains of the active center (acyl pocket and the hydrophobic patch), with the spatial differences between those sites presumably contributing to the inherent asymmetry of the AChE active center environment (Ordentlich *et al.*, 1999). The resulting stereoselectivity in particular with respect to methylphosphonofluoridates were utilized for in depth investigation of the hydrophobic and steric interactions with the structural elements of the active center (Ordentlich *et al.*, 1999). Early modeling studies attributed the 10^4-fold stereoselectivity of AChE toward the P^S-enantiomer of sarin mainly to the restrictive dimensions of the acyl pocket (Barak *et al.*, 1992). More recent studies with the diastereomers of soman suggest that a more complex array of interactions is affecting affinity and reactivity in the corresponding phosphylation reactions (Ordentlich *et al.*, 1999). In the case of phosphonofluoridates those interactions do not seem to include the leaving group and therefore interactions with the fluorine do not contribute to AChE stereoselectivity toward soman.

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* Numbers in parentheses refer to positions of analogous residues in TcAChE according to the recommended nomenclature (Massoulie *et al.*, 1992)

Interaction of the AChE active center with OP agents bearing positively charged leaving group is characterized by additional polar interaction that should affect the affinity as well as the stereoselectivity toward these inhibitors (Hosea *et al.*, 1996; Shafferman *et al.*, 1998; Kovarik *et al.*, 2003,). Indeed, AChE inhibition studies with various charged and noncharged O-ethyl methylphosphonothioates demonstrated the contribution of such polar interactions to the reactivities of the respective inhibitors (Kabachnik *et al.*, 1970). All those reactions result in the same phosphonylation product (O-ethyl methylphosphono-AChE) the structure of which has been recently determined by x-ray crystallography, following AChE phosphonylation by VX (Millard *et al.*, 1999). Also, AChE stereoselectivity toward certain O-alkyl S-[(trimethylamino)ethyl] methylphosphono-thioates was found to be much lower than that found for analogous phosphonofluoridates (Hosea *et al.*, 1996; Shafferman *et al.*, 1998). These studies indicated also that the thiocholine leaving group of these inhibitors is oriented toward the gorge entrance and that residue Asp74 is a primary determinant in AChE specificity for cationic organophosphorus agents (Hosea *et al.*, 1996). This finding is quite surprising in view of the x-ray structures of AChE in complex with noncovalent inhibitors like edrophonium (Harel *et al.*, 1993) or conjugated with covalent modifiers like TMTFA (Harel *et al.*, 1996) that show invariably that the ammonium cationic head points toward the anionic subsite residue Trp86 and being quite remote from Asp74.

In the present report we provide data regarding the origins of HuAChE stereoselectivity toward the enantiomers of VX determined by comparison with their noncharged isosteres as well as with the symmetrical diethyl phosphates echothiophate and its noncharged analogue. Our results indicate that in the case of VX stereoselectivity is a result of multiple interactions involving several elements of the active center and the peripheral anionic site, including polar interactions with the charged leaving group.

RESULTS

Preparation of organophosphate inhibitors: Resolved O-ethyl methylphosphonothioic acids, which served as starting materials for both the VX and the nc-VX enantiomers (Figure 8), were synthesized following the procedure of Berman and Leonard (1989). S- alkylation of the resolved enantiomers, using N,N-diisopropylaziridinium chloride, yielded pure VX enantiomers. This procedure was found more convenient than that using N,N-diisopropylaminoethyl chloride, as reported earlier (Hall *et al.*, 1977). Analogous preparation of the nc-VX enantiomers was carried out using 3-(2-bromoethyl)-2,4-dimethylpentane as the alkylating agent. The resulting nc-VX were obtained >95% enantiomerically pure as shown by ^{1}HNMR using the chiral reagent [R]-2,2,2-trifluoro-1-(9-anthryl)ethanol. O,O-diethyl S-(3,3-dimethylbutyl) phosphorothioate ("nc-echothiophate") was prepared from 3,3-dimethylbutyl bromide (Ordentlich *et al.*, 1993) and potassium salt of diethyl thiophosphoric acid.

Reactivity of HuAChE toward VX and nc-VX enantiomers: Reactivity of recombinant wild type HuAChE toward VX^S was 115-fold higher than toward the VX^R with the respective values of the bimolecular rate constants being $1.4x10^8$ $min^{-1}M^{-1}$ and $1.2x10^6$ $min^{-1}M^{-1}$. Such HuAChE stereoselectivity toward VX enantiomers is consistent with earlier reports on AChE reactivities toward

VX (Hall *et al.*, 1977, Benschop *et al.*, 1988) and toward other charged methylphosphonothiocholines (Hosea *et al.*, 1996, Berman *et al.*, 1989). In particular, AChE from bovine erythrocytes was reported to display a 200- fold stereoselectivity toward the VX^S enantiomer (Benschop *et al.*, 1988)

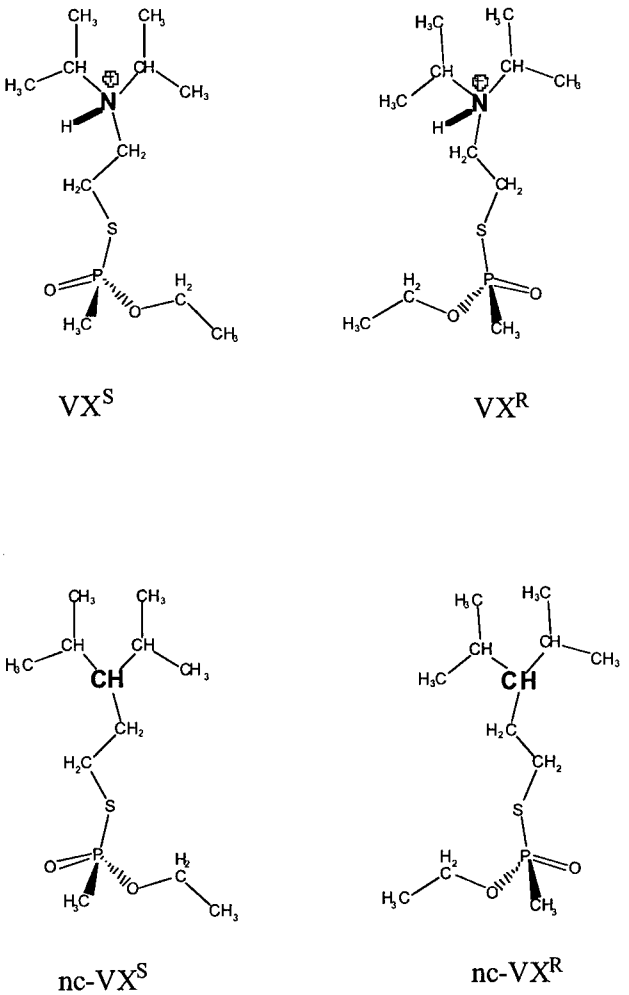


Figure 8: Chemical formulae of VX enantiomers and their noncharged isosteres. The protonated forms of both VX^S and VX^R are shown since the charged species are present under our experimental conditions (pK_a value for VX was recently reported to be 9.4, see Van der Schans, *et al.*, 2003)

The HuAChE enzyme displayed 60-fold stereoselectivity toward the noncharged nc-VX^S (Figure 8), with reactivity toward this enantiomer being about 12500-fold lower than that toward VX^S (Tables 2,3). The corresponding reactivity toward the nc-VX^R was exceedingly low (the value bimolecular rate constant k_i was $2x10^2$ $min^{-1}M^{-1}$) and the actual measurements of this constant, as well as of those for other HuAChE enzymes, was made possible only by the stability of the inhibitors in buffer solutions, and by the ability to produce sufficient amounts of recombinant enzyme proteins, using the remarkably efficient HEK 293 expression system (Shafferman *et al.*, 1992, Kronman *et al.*, 1992).

Table 2: Apparent bimolecular rate constants[a] (k_i) of phosphonylation of HuAChE enzymes by VX^S and VX^R

	VX^S		VX^R		
HuAChE	k_i ($x10^{-4}x$ $M^{-1}xmin^{-1}$)	**WT/ mutant**	k_i ($x10^{-4}x$ $M^{-1}xmin^{-1}$)	**WT/ mutant**	**VX^S/VX^R**
Wild Type	13700	1	118.0	1	115
F295A	1600	9	360.0	0.3	5
F297A	1560	9	22.5	5	70
F295A/F297A	18	760	3.4	35	5
W86F	465	30	10.0	12	50
W86A	3	4500	0.1	980	30
D74N	107	130	48.0	2.5	2
D74E	5900	2	31.0	4	190
W286A	13000	1	85.0	1	170
Y341A	5100	3	13.0	9	370
Y337A	4000	3	30.0	4	130
F338A	2700	5	30.0	4	90
G122A	7	2000	0.3	400	25

[a]Values represent mean of triplicate determinations with standard deviation not exceeding 20%.

Table 3: Apparent bimolecular rate constants[a] (k_i) of phosphonylation of HuAChE enzymes by nc-VX^S and nc-VX^R

	nc-VX^S		nc-VX^R		
HuAChE	k_i ($x10^{-4}x$ $M^{-1}xmin^{-1}$)	**WT/ mutant**	k_i ($x10^{-4}x$ $M^{-1}xmin^{-1}$)	**WT/ mutant**	**VX^S/VX^R**
Wild Type	1.1	1	0.02	1	60
F295A	0.5	2	0.03	1	20
F297A	3.4	0.3	0.02	1	170
F295A/F297A	0.04	27	0.003	7	13
W86F	0.6	1.8	0.025	1	25
W86A	0.3	3.7	0.01	2	30
D74N	2.0	0.5	0.03	1	70
D74E	0.9	1	0.01	2	90
W286A	1.5	0.7	0.015	1	100
Y341A	2.5	3	0.02	1	23
Y337A	63.0	0.03	0.07	0.3	900
F338A	0.9	1	0.003	7	300
G122A	0.02	60	0.0004	50	50

[a]Values represent mean of triplicate determinations with standard deviation not exceeding 20%.

Wild type HuAChE displayed a 850-fold higher reactivity toward the diethyl phosphate echothiophate than toward its noncharged isostere nc-echothiophate (see Table 4). Since for both phosphates disposition of the phosphyl ethoxy substituents with respect to the HuAChE active center should be similar, the different reactivities signify the contribution of interactions with the charged leaving group. Thus the large reactivity differences between the VX enantiomers and their corresponding noncharged isosteres (nc-VX) may have also resulted predominantly from accommodation of the diisopropylammonium group in the active center.

Table 4: Apparent bimolecular rate constants[a] (k_i) of phosphonylation of HuAChE enzymes by Echothiophate and nc-Echothiophate

	Echothiophate			nc-Echothiophate		
HuAChE	k_i ($x10^{-4}$x $M^{-1}xmin^{-1}$)	k_2 (min^{-1})	K_i ($x10^{-6}M$)	k_i ($x10^{-4}$x $M^{-1}xmin^{-1}$)	k_2 (min^{-1})	K_i ($x10^{-6}M$)
Wild Type	154	3.7	2.4	0.18	0.6	325
F295A	6000	0.8	0.0013	1.46	0.95	65
F297A	58	4.4	7.6	0.2	0.3	146
F295A/F297A	45	ND[b]	ND	0.02	ND	ND
W86F	17	0.7	4.2	0. 25	0.56	220
W86A	0.08	0.8	1020	0.02	0.8	4570
D74N	3.8	5.5	147	0.96	0.55	57
D74E	32	1.1	3.4	0.18	0.92	510
D74G	14	0.9	3.2	0.72	0.6	83
W286A	260	1.7	0.64	1.07	0.6	55
Y341A	40	1.2	3.0	0.4	0.97	244
Y337A	34	1.8	5.5	0.06	0.34	540
F338A	12	1.7	14.1	0.009	0.13	1500

[a] Values represent mean of triplicate determinations with standard deviation not exceeding 20%.

[b] Not determined

While interactions with the charged leaving group seem to consist the major contribution to the HuAChE affinity toward the two VX enantiomers, the question regarding the specific interactions contributing to the 115-fold stereoselectivity toward VX^S remains open. Since stereoselectivity has usually been associated with perturbations at the acyl pocket (Ordentlich *et al.*, 1993, 1999, Barak *et al.*, 1992), the lower reactivity of HuAChE toward VX^R may still be a result of inferior accommodation of the ethoxy substituent. Comparison of the bimolecular rate constant (k_i) for HuAChE phosphonylation by VX^R and by echothiophate shows quite similar values ($1.2x10^6$ $min^{-1}M^{-1}$ and $1.5x10^6$ $min^{-1}M^{-1}$ respectively), suggesting that such interactions of the ethoxy moiety may indeed determine the affinities of these two charged organophosphorus inhibitors. On the other hand, HuAChE reactivity toward nc-echothiophate was found to be only 6-fold lower than toward nc-VX^S,

indicating that replacement of a methyl group by ethoxy group in the acyl pocket results roughly in a 6-fold decrease in binding affinity. This effect seems too low to account for the observed HuAChE stereoselectivity toward VX^S.

Therefore it was important to investigate the dependence of stereoselectivity toward VX enantiomers on interactions with the different components of the active center functional architecture. To this end, enzyme derivatives with modifications introduced by site directed mutagenesis at active center subsites involved in ligand accommodation such as the acyl pocket, anionic subsite, hydrophobic subsite, peripheral anionic site and the loop of the oxyanion hole, were introduced. Kinetic evaluation of the reactivity characteristics of these HuAChE enzymes toward the charged and non-charged VX as well as toward the symmetric phosphate echothiophate allowed for characterization of the main elements of the HuAChE chirality toward charged methylphosphonates.

Stereoselectivity of HuAChE enzymes carrying mutations at the acyl pocket: Replacements of the acyl pocket residues Phe295 or Phe297 by alanine had quite different effects on stereoselectivity toward the VX^S enantiomer. While the F295A enzyme was only 5-fold more reactive toward the VX^S than toward the VX^R enantiomer, the corresponding ratio for the F297A was 70-fold, resembling that of the wild type HuAChE (see Table 2). The decrease in stereoselectivity of the F295A enzyme resulted mainly from its lower reactivity toward VX^S (9-fold) rather than from increased reactivity toward VX^R (3-fold, see Table 2). Thus, while structural perturbation at position 295 of the acyl pocket affects stereoselectivity toward the VX^S enantiomer, the basis for the differential reactivity does not seem to involve steric exclusion from the acyl pocket. The notion that steric congestion at the acyl pocket does not determine HuAChE stereoselectivity toward VX^S is supported also by reactivities of the F297A enzyme toward the VX enantiomers. In this case the mutated enzyme is less reactive toward both VX^S (9-fold) and VX^R (5-fold), suggesting a suboptimal accommodation of the phosphonate in the active center irrespective of the substituent adjacent to the acyl pocket

Mutations at positions 295 and 297 in HuAChE had only a small effect on reactivity of the corresponding enzymes toward the nc-VX enantiomers (all effects were within factor 3, see Table 3). These findings seem to suggest that there is nearly no energetic penalty for accommodating the nc-VX^R ethoxy substituent in the HuAChE acyl pocket.

Replacement of the aromatic residue at position-295 resulted in a 40-fold reactivity increase toward the diethyl phosphate - echothiophate, relative to the wild type enzyme. Corresponding replacement at position-297 had practically no effect, suggesting that orientation of the ethoxy group with respect to the acyl pocket in the echothiophate-HuAChE complex is somewhat different from that of the corresponding complex with the VX^R enantiomer. This different reactivity toward echothiophate may have to do with the other ethoxy substituent since similar effects, due to mutations at the acyl pocket, were reported for neutral diethyl phosphates like DEFP and paraoxone (Ordentlich *et al.*, 1996).

The double mutant F295A/F297A exhibits lower affinity toward both VX and nc-VX, with consistently larger effects for the P^S enantiomers (see Tables 2, 3). Interestingly, the reactivity decline toward VX^S is much more pronounced than that toward nc-VX^S (760-fold and 27-fold respectively), suggesting that the reactivity decrease does not seem to be a result of deficient accommodation of the

methyl group. It appears also that the outcome of removing aromatic moieties from the acyl pocket, (e.g. enhanced mobility of the catalytic His447 see Kaplan *et al.*, 2001, 2004; Barak *et al.*, 2002) has larger effect on the presumably more rigidly oriented VX^S within the Michaelis complex as compared to the neutral nc-VX^S.

Stereoselectivity of HuAChE enzymes carrying mutations at the anionic subsite – choline binding site: Replacement of residue Typ86 by alanine had a dramatic effect on the reactivity toward both VX enantiomers, yet only a minor effect on stereoselectivity toward the VX^S (Table 2). The decrease of the W86A HuAChE reactivity toward the VX^S and VX^R enantiomers as compared to the wild type enzyme (the values of bimolecular rate constants decreased by 4500-fold and almost 1000-fold respectively, see Table 2) was compatible with what was observed in the past for other covalent or noncovalent charged ligands (Ordentlich *et al.*, 1993, 1995; Ariel *et al.*, 1998; Kabachnik *et al.*, 1970; Millard *et al.*, 1999; Shafferman *et al.*, 1992b). The corresponding effects of the replacement of Trp86 by phenylalanine were much smaller and consistent with the well established role of the aromatic residue at position - 86 as the main interaction locus of the charged moieties of AChE ligands (Ordentlich *et al.*, 1995; Ariel *et al.*, 1998). Similarly, W86A was 1925-fold less reactive toward echothiophate than the wild type enzyme (Table 4). These effects are considerably higher than those reported for interaction of similar charged methylphosphonates, P^S-O-cycloheptyl methylphosphonylthiocholine, (40-fold) or the corresponding P^R- enantiomer (33-fold) with W86A mutant of the mouse AChE (Hosea *et al.*, 1996).

Mutations at the anionic subsite had only a minor effects on HuAChE affinities toward the nc-VX enantiomers and the nc-echothiophate (Tables 3, 4), demonstrating again that the role of the aromatic moiety at position-86 consists mainly of accommodating the charged moieties of substrates and other charged ligands. However, reactivity of the W86A HuAChE toward nc-VX^S was still 10-fold lower than that toward VX^S, suggesting participation of other elements in the active center in accommodation of the charged leaving group. Similarly, reactivity of this enzyme toward nc-echothiophate was 4-times lower than that toward echothiophate (Table 4).

Stereoselectivity of HuAChE enzymes carrying mutations at the hydrophobic subsite: Stereoselectivity toward VX^S as well as reactivity toward both VX enantiomers is only slightly affected by single replacements of elements of the hydrophobic pocket (aromatic patch, (Ariel *et al.*, 1998) residues Tyr337 and Phe338 (Table 2). The effects are small (~4-fold) and do not indicate disruption of major interactions with the active center environment. In view of this result it was rather surprising to find that replacement of this residue by alanine resulted in a 57-fold increase in affinity toward nc-VX^S, suggesting that the S-alkyl leaving group of nc-VX^S appears to interfere with the aromatic moiety of Tyr337 (Table 3). A small increase in affinity is observed also for the nc-VX^R and consequently the stereoselectivity of this enzyme toward nc-VX^S was ~900-fold. Such effect on stereoselectivity due to replacement of Tyr337 indicates that the leaving groups in VX and in nc-VX may point to somewhat different regions of the active site. Furthermore, the Y337A HuAChE was only 3-fold less reactive toward the nc-echothiophate than the wild type enzyme (Table 4), implying that the orientation of its

leaving group, in the nc-echothiophate-Y337A complex, resembles more that of nc-VX^R than that of the VX^S (see Table 3). The findings indicate also that the positively charged leaving groups of either the VX enantiomers or echothiophate do not interact with the aromatic moiety of Tyr337 (Table 2, 4). In this respect the findings are consistent with our previously reported conclusion that residue Tyr337 is not a part of the anionic subsite participating in accommodation of the cationic head groups of AChE ligands (Ariel *et al.*, 1998), as suggested before (Harel *et al.*, 1996; Greenblatt *et al.*, 1999).

Stereoselectivity of HuAChE enzymes carrying mutations at the peripheral anionic site (PAS): Replacement of Asp74 by asparagine practically abolished stereoselectivity toward the VX^S enantiomer. The D74N HuAChE was 130-fold less reactive toward VX^S while its reactivity toward VX^R resembled that of the wild type enzyme (see Table 2). In contrast, substitution of the aspartate at position 74 by another acidic residue glutamate, yielded an enzyme with nearly the same affinities toward both VX enantiomers as the wild type HuAChE. These results show that VX^S interacts with the acidic residue at position 74 of HuAChE, suggesting that in the case of VX enantiomers charged interactions of the leaving group are a major determinant of stereoselectivity. Thus, it is reasonable to expect that the charged leaving groups in the HuAChE Michaelis complexes of VX^S and VX^R assume somewhat different orientation, with the cationic head in the VX^S complex closer to the Asp74 carboxylate than in the corresponding VX^R complex.

Reactivity of the D74N HuAChE toward echothiophate was also 40-fold lower than that of the wild type enzyme (see Table 4), yet in this case the decrease may not result from loss of a specific electrostatic interaction of the cationic leaving group. This notion is suggested by the similar effects of replacing residue Asp74 by glutamic acid or by the noncharged residue glycine (5-fold and 10-fold respectively, see Table 4 and Shafferman *et al.*, 1998).

Replacement of Asp74 by asparagine had practically no effect on the reactivity toward nc-VX enantiomers (see Table 3). In addition, a minor (4-5-fold) increase was observed in the activity of D74N and D74G HuAChEs toward nc-echothiophate (Table 4). These findings are consistent with our previous observation that residue Asp74 contributes predominantly to binding of charged ligands in the HuAChE active center (Shafferman *et al.*, 1992b, 1998).

Replacement of other components of the PAS Tyr341 and Trp286 had only limited effect on reactivity toward VX enantiomers. The most pronounced effect was that due to substitution of position 341 by alanine on reactivity toward VX^R (9-fold). Also, these replacements had nearly no effect on affinities toward nc-VX enantiomers or nc-echothiophate (see Tables 2-4).

Stereoselectivity of HuAChE carrying mutation at position 122 next to the oxyanion hole: Residue Gly122 is adjacent to the oxyanion hole residues Gly120 and Gly121. Replacement of Gly122 by alanine was shown to introduce a methyl group into the space of the acyl pocket (Ordentlich *et al.*, 1998, see also Harel *et al.*, 1996). As already reported, the reactivity of G122A toward phosphonates is affected to a larger extent than toward phosphates (Ordentlich *et al.*, 1998), due to the size of the moiety in the immediate vicinity of the Ala122 methyl group (e.g. for DEFP reactivity of the G122A HuAChE was 95-fold lower than for the wild type HuAChE and for soman the corresponding ratio

was 500; Ordentlich *et al.*, 1998). In the case of VX^S the G122A HuAChE was 2000-fold less reactive, compared to the wild type enzyme while for VX^R the corresponding ratio is 400 (Table 2). Thus, G122A HuAChE displayed lower stereoselectivity, than the wild type enzyme, toward the VX^S enantiomer (25-fold) probably due to impaired accommodation of the phosphonyl methyl group in the acyl pocket as a consequence of replacing the hydrogen by a methyl group in the G122A mutant. The G122A enzyme was also 260-fold less reactive toward echothiophate compared to the wild type HuAChE (k_i = $6x10^3xM^{-1}xmin^{-1}$), suggesting that in the phosphate-G122A adduct the phosphate ethoxy group may be positioned in the acyl pocket more like that of VX^R (see Table 2) than like that of the homologous phosphate DEFP (Ordentlich *et al.*, 1998). These observations may indicate that in the Michaelis complexes of both VX enantiomers or of echothiophate the methyl and the ethoxy moieties are located closer to Ala122 than in the corresponding complexes of DEFP or soman. Such proximity, which may be induced by the rigid orientation of the charged leaving group, suggests also that the corresponding phosphyl moieties are positioned closely to the oxyanion hole.

The reactivity of the G122A HuAChE toward the nc-VX^S enantiomer was 60-fold lower than that of the wild type enzyme (see Table 3). Such reactivity decrease is much smaller than could be expected from comparison to the corresponding ratios for both VX^S and soman (2000-fold and 500-fold respectively), again suggesting that accommodation of the nc-VX^S in HuAChE active center is quite different from that of the other phosphonates.

Molecular modeling of Michaelis complexes of HuAChE with VX enantiomers: The question of AChE stereoselectivity toward VX^S has already been addressed in the past by molecular dynamics simulations of the TcAChE-VX Michaelis complexes (Albaret *et al.*, 1997). In construction of the initial structures it was assumed that the cationic leaving group in both the VX^S and the VX^R complexes was oriented toward the peripheral anionic site and that the electrostatic interaction with Asp(72) contributes substantially to the accommodation of the charged diisopropylammonio moiety (Hosea *et al.*, 1996). Such assumption was consistent with the role played by residue Asp(72) in accommodating the charged groups of certain methylphosphonothiocholines in the Michaelis complexes with TcAChE (Hosea *et al.*, 1996). The simulations suggested that TcAChE stereoselectivity toward VX enantiomers resulted from impaired interaction of the VX^R with the oxyanion hole subsite (Albaret *et al.*, 1997). Our results, from kinetic studies with the wild type and the G122A enzymes, suggest however that in HuAChE both VX enantiomers appear to be well accommodated in the oxyanion hole. Furthermore, interactions with oxyanion hole seem to be crucial for HuAChE reactivity toward phosphate inhibitors and therefore a significant misalignment of the P=O moiety with respect to this subsite could abolish the phosphylation process (Ordentlich *et al.*, 1998). Thus it appeared interesting to examine the dynamic behavior of the HuAChE-VX complexes, especially in view of the finding, from kinetic studies, regarding the role of residue Asp74 in determining HuAChE stereoselectivity toward VX (Table 2).

In the present study molecular simulations of the HuAChE-VX complexes were performed assuming that proper orientation and proximity of the phosphorus atom to O^g-Ser203 atom are essential for the phosphylation process and therefore should be kept throughout the simulation. In addition, interaction

distances of the phosphyl oxygen from the oxyanion hole elements were maintained since polarization of the P=O bond was found to be of a crucial importance to subsequent chemical process. Therefore during optimization of the initial structures and the dynamic simulation runs the positioning of the VX P=O moieties was restrained with respect to the HuAChE active center. In the two diastereomeric initial structures of the VX-HuAChE Michaelis complexes the protonated diisopropylammonio group was juxtaposed with the anionic subsite Trp86 (see Table 5). Simulation experiments showed that for the VX^R complex there was no significant change in the positioning of the cationic head with respect to residues Trp86 and Asp74 (Figure 9B). On the other hand, in the VX^S complex there is motion of the cationic head toward the gorge exit, shortening its distance from residue Asp74 (see Figures 9A, 9C). Examination of the structures along the simulation trajectories suggested that orientation of the cationic leaving group was influenced by its interaction with the phosphonyl substituent pointing toward the acyl pocket. In the VX^S complex the phosphonyl methyl group is accommodated in the acyl pocket permitting the cationic leaving group to point toward the gorge entrance and to interact with both residues Trp86 and Asp74 (Figure 9A). On the other hand, in the VX^R complex the ethoxy group is forced to point upward by the restricted space of the acyl pocket (see Figure 9B). Such conformation of the ethoxy moiety interferes with the cationic leaving group inducing an alternative conformation where the diisopropylammonio group is displaced away from residue Asp74 (and toward residue Trp86), as compared to its orientation in the VX^S complex.

Table 5: Changes of relevant distances in the HuAChE Michaelis complexes of VX^S and VX^R following molecular dynamics simulation.

Distance	*Initial Structures (Å)*		*Changes in Average simulated structures (ΔÅ)*	
	VX^S	**VX^R**	**VX^S**	**VX^R**
$N_{G120} - O(=P)_{VX}$	2.97	2.98	0.12	0.22
$N_{G121} - O(=P)_{VX}$	2.88	3.18	-0.06	-0.21
$N_{A204} - O(=P)_{VX}$	3.40	3.10	0.27	0.10
$O^{\gamma}{}_{S203} - P_{VX}$	3.33	3.34	-0.03	0.16
$O^{\delta 1}{}_{D74} - N_{VX}$	7.30	7.69	-1.10	0.41
$C^{\delta 2}{}_{W86} - N_{VX}$	5.10	5.22	1.18	0.15

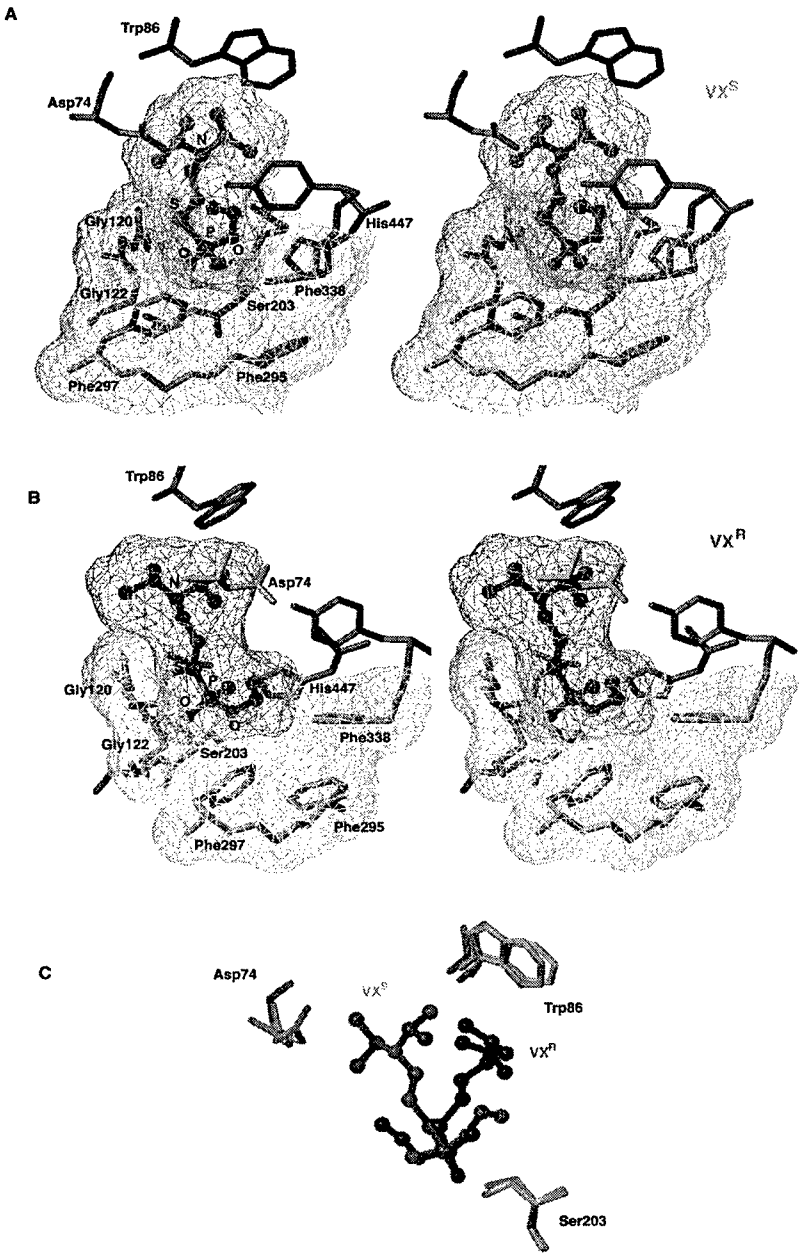


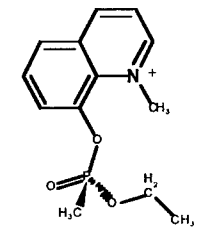
Figure 9: Average structures from molecular dynamics simulations of Michaelis complexes of the VX^S and VX^R enantiomers with HuAChE. Only amino acids adjacent to the inhibitor are shown and hydrogen atoms are omitted for clarity. Interatomic distances defining the relative orientation of the respective ligands are listed in Table 4. (A) Stereoview of the VX^S-HuAChE complex with the phosphonate depicted in magenta and the protein in green. The volumes of the VX^S and the molecular environment around the acyl pocket are shown as grid. (B) Stereoview of the VX^R-HuAChE complex with the phosphonate depicted in red and the protein in cyan. The volumes of the VX^R and the molecular environment around the acyl pocket are shown as grid. (C) Superposition of the VX^S-HuAChE and VX^R-HuAChE complex structures, using the Cα atoms of the residues shown and the respective phosphorus atoms as reference points. Note that the ammonio group of the VX^S complex (shown in magenta) is proximal to both Asp74 carboxylate and the indole moiety of Trp86, while that of the VX^R complex (shown in red) points exclusively in the direction of residue Trp86.

DISCUSSION

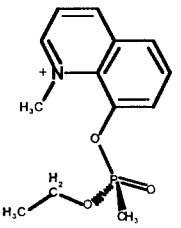
Early hypotheses (Jarv , 1984) and modeling experiments (Barak *et al.*, 1992) related the pronounced stereoselectivity of AChE toward methylphosphonofluoridates like sarin or soman to the structure of the acyl pocket. The main reason for this assessment was that the homologous enzyme butyrylcholinesterase (BChE), in which residues at positions corresponding to 295 and 297 are isoleucine and valine respectively (Harel *et al.*, 1992), was found to be more reactive than AChE toward bulky substrates like butyrylcholine (BCh) and organophosphorus inhibitors like diisopropyl phosphorofluoridate (DFP) as well as not stereoselective toward sarin (Ordentlich *et al.*, 1993, 1999; Vellom *et al.*, 1993; Hosea *et al.*, 1995; Boter *et al.*, 1971). Indeed, studies of HuAChE reactivity using site directed mutagenesis and enzyme kinetics suggested that the acyl pocket residues Phe295 and to a lesser extent Phe297 determine specificity toward acylating (substrates) and phosphylating agents (Ordentlich *et al.*, 1993, 1996) mainly by limiting the volume of the acyl pocket. A more recent examination of stereoselectivity in reactions of HuAChE active center mutants with diastereomers of soman, suggested that while the AChE acyl pocket is in fact the main determinant of the relative reactivity toward the P^S- and the P^R- diastereomers, the actual mechanism of stereoselectivity is only partially related to steric interference (Ordentlich *et al.*, 1999). Further inquiry into the consequences of structural modifications at the HuAChE acyl pocket led us to propose that residues Phe295 and Phe297 are also part of an aromatic system involved in "trapping" the catalytic His447 (Kaplan *et al.*, 2001; Barak *et al.*, 2002). Thus, modifications of the AChE acyl pocket may perturb the positioning of His447 and thereby impair the accommodation of tetrahedral species in the active center. Such modifications of the acyl pocket could be brought about either by accommodation of bulky groups, leading to a significant relocation of the side chains of both Phe295 and Phe297, or by replacement of these residues by aliphatic amino acids (Kaplan *et al.*, 2001, 2004; Barak *et al.*, 2002).

Structural characteristics of the acyl pocket seem also to play a role in determining HuAChE stereoselectivity toward VX^S, as demonstrated by the mere 5-fold stereoselectivity of the F295A and the F295A/F297A enzymes (see Table 2). For the latter, the 760-fold reactivity decrease toward the VX^S enantiomer was accompanied by only a 35-fold reactivity decline toward the VX^R. Apparently the overall effect of the double mutation at the acyl pocket, on reactivity toward VX^R, was smaller due to some compensating factor. Such compensation could come from relief of steric pressure (due to removal of the bulky aromatic residues) on the ethoxy substituent in the acyl pocket. Yet, there is no evidence that accommodation of an ethoxy group results in perturbation of the acyl pocket. In fact, the lack of AChE stereoselectivity toward MEPQ (see Figure 10), where all the phosphorus substituents apart from the charged leaving group are identical to those of VX (Levy and Ashani 1986), indicates that the acyl pocket accommodates equally well the methyl and the ethoxy substituents. Therefore, the different effects, due to modifications of the acyl pocket, on reactivity toward the VX^S and the VX^R enantiomers have to originate from interactions with other elements of the active center. Indeed, for both the VX^S and the VX^R enantiomers interactions of the charged leaving group appear to be another major determinant of HuAChE stereoselectivity. The findings that the D74N HuAChE was practically devoid of stereoselectivity toward VX^S and yet reactivity of this mutant toward VX^R was nearly

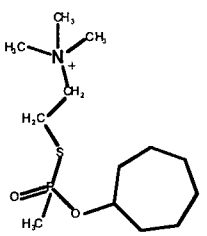
equivalent to that of the wild type enzyme, indicated that the difference in HuAChE accommodation of the two VX enantiomers can be reduced to a single interaction of the charged phosphyl substituent with residue Asp74. Thus, while the diisopropylammonium moiety in Michaelis complexes of both VX^S and VX^R enantiomers interacts mainly with the anionic subsite residue Trp86, as demonstrated by the respective 980-fold and 4500-fold decreases in reactivity toward the W86A enzyme (Table 2), in the HuAChE-VX^S complex additional stabilization is provided by its interaction with the carboxylate of residue Asp74. In fact, interaction of the thiocholine leaving groups of certain O-alkyl S-[(trimethylamino)ethyl] methylphosphonothioates with residue Asp74 has already been suggested (Hosea *et al.*, 1996). However for these ligands both enantiomers interacted with Asp74 and their interaction with Trp86 was less pronounced.



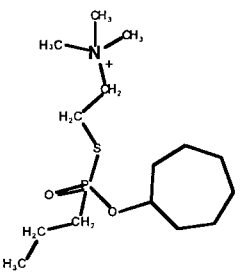
$MEPQ^S$



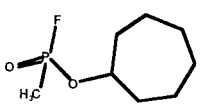
$MEPQ^R$



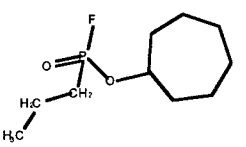
CHMPT



CHPPT



CHMPF



CHPPF

Figure 10: (A) Structural formulae of the MEPQ enantiomers which display nearly equivalent reactivity toward AChE (Levy and Ashani, 1986) while yielding the same adducts as the corresponding enantiomers of VX. (B) Formulae of O-cycloheptyl alkylphosphonate AChE inhibitors bearing thiocholine and fluorine as leaving groups (Berman, 1995). Note that while reactivities of the two methyl phosphonates CHMPT and CHMPF are similar, reactivity of CHPPT is much lower than that of CHPPF (see Berman, 1995 and text)

If stabilizing interaction of the diisopropylammonium moiety with Asp74 is possible in the VX^S Michaelis complex why is it absent in the corresponding VX^R complex. The reason for that is suggested by the molecular simulation experiments, which show the cationic moiety of VX^S to be located nearly 2 Å closer to the Asp74 carboxylate than the corresponding moiety of VX^R (see Table 5). Thus the respective locations of the VX cationic moieties seem to be determined by interactions with the phosphonyl ethoxy substituent. In the HuAChE-VX^S complex the ethoxy group, positioned in the hydrophobic pocket, allows for an apparently optimal juxtaposition of the diisopropylammonium group with respect to residues Trp86 and Asp74 (Figure 9A). On the other hand, in the HuAChE-VX^R complex, the ethoxy group emerging from the acyl pocket, induces an alternative conformation of the leaving group, in which interaction with residue Asp74 is practically precluded (Figure 9B). According to this molecular scenario opening of the acyl pocket, as in the F295A/F297A HuAChE, may relieve the steric crowding around the VX^R phosphorus , allowing for interaction of the cationic moiety in the corresponding Michaelis complex with the carboxylate of Asp74.

To our knowledge, the notion that *intramolecular* interactions of the phosphyl moiety may contribute to AChE stereoselectivity toward VX^S or any other organophosphate inhibitor was not considered before. Yet certain results reported in the past seem consistent with the suggestion that interactions of charged leaving groups with other bulky substituents affect the inhibition properties of the corresponding phosphates and phosphonates. One such example is the different accommodation, by AChE, of n-propylphosphonates bearing either fluorine or thiocholine as a leaving group (Berman 1995), as compared to the respective methylphosphonates (see Figure 10). While cycloheptyl n-propylphosphonofluoridate (CHPPF) was only ~2-fold less reactive than the corresponding methylphosphonofluoridate (CHMPF) the cycloheptyl n-propylphosphonothiocholine (CHPPT) was found to be 220-fold less reactive than the methylphosphonothiocholine (CHMPT). Since reactivities of the two cycloheptyl methylphosphonates were quite similar ($6.8x10^8$ $min^{-1}M^{-1}$ and $1.4x10^8$ $min^{-1}M^{-1}$ for CHMPF and CHMPT respectively), the 360-fold lower reactivity of CHPPT, relative to the corresponding fluoridate (CHPPF), may indicate mutual interference of the two bulky phosphyl substituents. The resemblance of the reactivity ratio between CHPPT and CHMPT (220-fold) and the stereoselectivity observed here toward the VX enantiomers (115-fold, see Table 2) may not be accidental, since the overall dimensions of propyl and ethoxy substituents are quite similar. Therefore the relative reactivity decline for both the VX^R and the propylphosphonothiocholine may originate from similar interactions between the phosphonyl substituents.

The HuAChE reactivities toward echothiophate and its noncharged isostere nc-echothiophate is also consistent with the idea that the charged bulky leaving group affects the orientation of the ethoxy substituents within the active center. The value of the dissociation constant K_d for the HuAChE-echothiophate Michaelis complex is practically equivalent to that of the corresponding complex with DEFP ($2.4x10^{-6}$ M and $1.9x10^{-6}$ M respectively, (Ordentlich *et al.*, 1996), despite the fact that in the latter there is no stabilization due to charge interactions. The fact that hydrophobic interactions due to the ethoxy moieties, which stabilize the DEFP complex, do not seem to contribute to accommodation of the echothiophate complex can be understood by assuming that these moieties are improperly

oriented with respect to the active center of the enzyme, by interactions within the ligand. Furthermore, removal of the charged interaction, either through modification of the enzyme (aliphatic replacement at position 86 of HuAChE) or by utilizing the noncharged nc-echothiophate resulted in very low values of the respective bimolecular rate constants k_i (see Table 4). Thus, it appears that irrespective of charge, a large leaving group interferes with the hydrophobic stabilization of the HuAChE-diethyl phosphate complex.

The analysis presented above suggests that comparison of reactivities of the isosteric VX and nc-VX analogs toward HuAChE, in fact isolates the effect of charge on the relative affinity of the enzyme toward these methylphosphonothiolates. Without the charge interactions and in absence of significant contribution of the nc-VX hydrophobic substituents to accommodation in the active center, the reactivities of both nc-VX^S and nc-VX^R enantiomers were exceedingly low. Yet, HuAChE reactivity toward nc-VX^S was still 60-fold higher, as compared to that toward nc-VX^R, indicating presence of specific interactions underlying this HuAChE stereoselectivity. Although such interactions could not be fully characterized on the basis of the functional analysis described here, they appear to be different from those contributing to stereoselectivity toward VX^S. The largest effects observed are related to either introduction or removal of steric obstructions (e.g. effects due to replacement of Gly122 or Tyr337 respectively), rather to the effects related to perturbation at the acyl pocket (see Table 3).

The suggestion that interactions of the charged leaving group constitute the main determinant of HuAChE stereoselectivity toward VX^S may also provide an insight into the question regarding the wide range of AChE stereoselectivities toward different methylphosphonates. Namely, the outstanding stereoselectivity toward the P^S- diastereomers of soman (e.g. $7.5x10^4$-fold for the P^SC^S over the P^RC^S diastereomer, Ordentlich *et al.*, 1999), seems to be in contrast to that observed here, and in previous studies, for the VX^S (Benschop and De Jong 1988). The AChE stereoselectivity toward P^SC^S- and P^SC^R-soman diastereomers is also much higher than the stereoselectivity reported in the past for methylphosphonates carrying other leaving groups like p-nitrophenol (Benschop, *et al.*, 2003) or thiocholine (Hosea , *et al.*, 1996, Berman and Leonard 1989). These different stereoselectivities result predominantly from the exceedingly low reactivity of the P^R - diastereomers of methylphosphonofluoridates . For instance, while the AChE phosphonylation rate constant by VX^S ($1.37x10^8$ min^{-1} M^{-1} see Table 2) is similar to those measured for the P^SC^S- and P^SC^R diastereomers of soman ($1.5x10^8$ and $0.8x10^8$ $min^{-1}M^{-1}$ respectively; (Ordentlich, *et al.*, 1999), the corresponding constant for the VX^R ($1.18x10^6$ min^{-1} M^{-1}) is about 600-fold higher than those for the P^RC^S- or the P^RC^R-soman isomers ($2.0x10^3$ $min^{-1}M^{-1}$ for both cases). In fact, the reactivities of the P^R-soman diastereomers toward HuAChE resemble those of the nc-VX^S and nc-VX^R enantiomers, suggesting that hydrophobic interactions contribute little to the accommodation of the P^RC^S- or the P^RC^R-diastereomers of soman in the AChE active center.

In conclusion, it appears that HuAChE stereoselectivity toward methylphosphonates is determined by both the nature of the phosphonyl leaving group and the inherent asymmetry of the active center environment. The relative contribution of each of these elements seems to depend upon the nature of the specific inhibitor, with the active center asymmetry playing a dominant role in stereoselectivity toward P^S-soman while the positive charge of the leaving group being dominant in stereoselectivity

toward VX^S as well as toward other P^S-methylphosphonothiocholines. In contrast to the notion regarding the acyl pocket as the main component of the AChE active center asymmetry, it appears now that other subsites and in particular the PAS contribute to stereoselectivity of HuAChE toward VX and other methylphosphonates.

VI. KEY RESEARCH ACCOMPLISHMENTS

1. Evaluation of the synthetic human *ache* gene with altered codons (lower GC contents) under various transcription signals in *Bacillus brevis* bacterial cells.

2. Generation of a series of hypolysine AChEs, to allow the evaluation of different enzyme forms which may serve for the generation of homogenous PEG-conjugated circulatory long-lived OP bioscavengers.

3. Demonstration that substitution of one or two lysine residues does not significantly alter the enzymatic activity or thermostability of human AChE.

4. Generation of a multilysine-mutated human AChE enzyme exhibiting undiminished enzymatic activity and stability, for evaluation of its pharmacokinetic properties following PEGylation.

5. Demonstration that native BuChE has a circulatory residence time comparable to that of PEG-AChE.

6. Demonstration by *in-vitro* immunoprecipitation experiments, that PEG-conjugation of recombinant human AChE results in the generation of an enzyme form exhibiting significantly reduced antigenicity.

7. Demonstration that the PEG-conjugated enzyme (unlike non-modified recombinant human AChE) fails to elicit a significant antibody response even in a heterologous animal model.

8. Demonstration that HuAChE stereoselectivity toward VX enantiomers is practically abolished by replacement of the aromatic residues Phe295 and Phe297 of the acyl pocket as well as of the PAS residue Asp74, and that HuAChE interaction with the cationic moiety of is a major determinant of stereoselectivity toward VX enentiomers.

VII. REPORTABLE OUTCOME

List of publications related to the current contract

A., Shafferman, T., Chitlaru, A., Ordentlich, B., Velan, and C., Kronman (2004). A complex Array of Post-Translation Modifications Determines the Circulatory Longevity of AChE in a Hierarchical Manner. In: *Cholinergic Mechanisms*. Eds. I. Silman, H. Soreq, L. Anglister, D. Michaelson and A. Fisher (Taylor & Francis Publication), 245-253.

A.,Ordentlich, D., Barak, N., Ariel, C., Kronman, D., Kaplan, B., Velan and A., Shafferman (2004). Surprising findings from the functional analysis of Human AChE adducts of Alzheimer's disease drugs. In: *Cholinergic Mechanisms*. Eds. I. Silman, H. Soreq, L. Anglister, D. Michaelson and A. Fisher (Taylor & Francis Publication), 177-181.

O., Cohen, C., Kronman, T., Chitlaru, A., Ordentlich, B., Velan and A., Shafferman (2004). Generation of pharmacokinetically improved recombinant Human Acetylcholinesterase by polyethylene glycol modification. In: *Cholinergic Mechanisms*. Eds. I. Silman, H. Soreq, L. Anglister, D. Michaelson and A. Fisher (Taylor & Francis Publication), 519-521.

D., Kaplan, A., Ordentlich, D., Barak, N., Ariel, C., Kronman, B., Velan and A., Shafferman (2004). Attempts to engineer an enzyme-mimic of Butyrylcholinesterase by substitution of the six divergent aromatic amino acids in the active center of AChE. In: *Cholinergic Mechanisms*. Eds. I. Silman, H. Soreq, L. Anglister, D. Michaelson and A. Fisher (Taylor & Francis Publication), 601-605.

E., Elhanany, A., Ordentlich, O., Dgany, D., Kaplan, Y., Segall, R., Barak, B., Velan and A., Shafferman (2004). MALDI-TOF/MS analysis of tabun-AChE conjugate: A tool for resolution of "aging" pathway. In: Eds. I. Silman, H. Soreq, L. Anglister, D. Michaelson and A. Fisher (Taylor & Francis Publication), 563-565.

C., Kronman, T., Chitlaru, N., Seliger, S., Lazar, A., Lazar, L., Zilberstein, B., Velan and A., Shafferman (2004). Some basic rules governing oligosaccharide-dependent circulatory residence of glycoproteins are revealed by MALDI-TOF/MS mapping of the multiple N-glycans associated with recombinant bovine acetylcholinesterase. In: *Cholinergic Mechanisms*. Eds. I. Silman, H. Soreq, L. Anglister, D. Michaelson and A. Fisher (Taylor & Francis Publication), 613-616.

T., Chitlaru, C., Kronman, S., Lazar, N., Seliger, B., Velan and A., Shafferman (2004). Effect of post-translation modifications of human acetylcholinesterase on its circulatory residence. In: *Cholinergic Mechanisms*. Eds. I. Silman, H. Soreq, L. Anglister, D. Michaelson and A. Fisher (Taylor & Francis Publication), 511-514.

A. Ordentlich, D. Barak., G. Sod-Moriah, D. Kaplan, D. Mizrahi, Y. Segall, C. Kronman, Y. Karton, A. Lazar, D. Marcus, B. Velan, and A. Shafferman (2004) Stereoselectivity toward VX is determined by interactions with residues of the acyl pocket as well as of the peripheral anionic site of AChE. *Biochemistry* 43, 11255-11265.

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VIII. CONCLUSIONS

Examination of the pharmacokinetic performance of well-defined AChE forms differing in their post-translation processing demonstrated that the differently processed AChE forms displayed nearly equal circulatory residence time values subsequent to PEGylation, indicating that PEGylation of AChE overrides post-translation-related suboptimal processing. It therefore may be possible to utilize low-cost microorganisms-based production systems which do not support animal-cell-related post-translation modifications, for the production of recombinant human AChE at large scale. This microorganism-based recombinant enzyme product can then be converted into circulatory long-lived bioscavenger molecules by PEG-conjugation.

During the last year, we examined the possibility to express recombinant human AChE in *Bacillus brevis* cells, using a synthetic *ache* gene in conjunction with various potent transcription promoters. Our studies demonstrate that following tranformation, the bacterial cells synthesize authentic HuAChE. Detailed analysis demonstrated that the messenger RNA coding for human AChE is transcribed at sufficiently high levels and is relatively stable, however, the enzyme product is produced in low yield. At present, we are carrying out a series of experiments to determine whether this is due to low translation levels or to an instability of the AChE product. This will allow us to determine whether or not one can take advantage of the *B. brevis* expression system for production of human AChE.

The use of PEGylated AChE as a therapeutic agent would require that the enzyme product exhibit a high degree of homogeneity. This in turn, may require that some of the lysine residues which serve as targets for PEGylation be eliminated, to allow production of uniformly PEGylated enzyme. To this end, we began a series of studies aimed to determine the effect of lysine removal on enzyme functionality, pharmacokinetic performance and structural homogeneity following PEGylation.

During the course of the present year, we have established that substitution of any single lysine residue of human AChE, does not significantly compromise enzymatic performance or thermostability. Moreover, substitution of various lysine residue pairs also did not appreciably affect the kinetic performance of the enzyme nor its stability. Encouraged by the results from the double lysine mutant studies, we began the generation of multiple lysine mutants. To date, an enzymatically active thermostable multiple lysine mutant, K23A/K332A/K348A was generated. This multiple lysine mutant will be further examined (before and after PEGylation) for its pharmacokinetic performance and homogeneity. Delineation of the pertinent characteristics of these hypolysine AChE derivatives will provide us with the necessary guidelines for further adjustment of the lysine contents of the human enzyme product, to achieve an optimal combination of efficient bioscavenging activity and product uniformity.

Immunological studies of the non-modified and PEGylated human AChE allowed us to determine that the chemical modification of the enzyme indeed significantly decreased the antigenicity and immunogenicity of the enzyme. This result demonstrates that the conjugation of PEG to human AChE contributes to its therapeutic appropriateness not only by improving the pharmacokinetic performance

of the enzyme, but also by reducing immunological responses to the administered recombinant protein.

Finally, we investigated the stereoselectivity of human AChE toward the P^S enantiomer of the lethal chemical warfare agent VX, by examining the reactivity of HuAChE and its mutant derivatives toward purified enantiomers of VX and its noncharged isostere O-ethyl S-(3-isopropyl-4-methyl-pentyl) methylphosphonothioate (nc-VX) as well as echothiophate and its noncharged analogue. Reactivity of the wild type HuAChE toward VX^S was 115-fold higher than that toward VX^R, with the corresponding bimolecular rate constants $1.4x10^8$ $min^{-1}M^{-1}$ and $1.2x10^6$ $min^{-1}M^{-1}$. HuAChE was also 12500-fold more reactive toward VX^S than toward nc-VX^S. Substitution of the cation binding subsite residue Trp86 by alanine resulted in a 3-orders of magnitude decrease in HuAChE reactivity toward both VX enantiomers, while this replacement had only a marginal effect on the reactivity toward the enantiomers of nc-VX and the noncharged echothiophate. These results attest to the critical role played by Trp86 in accommodating the charged moieties of both VX enantiomers. Marked decrease in stereoselectivity toward VX^S was observed following replacements of Phe295 at the acyl pocket (F295A and F295A/F297A). Replacement of the peripheral anionic site (PAS) residue Asp74 by asparagine (D74N) practically abolished stereoselectivity toward VX^S (130-fold decrease), while substitution which retains the negative charge at position 74 (D74E) had no effect. The results from kinetic studies and molecular simulations suggest that the differential reactivity toward the VX enantiomers is mainly a result of a different interaction of the charged leaving group with residue Asp74. Thus, the combined mutagenesis and kinetics studies with various derivatives of VX allowed us to resolve on a molecular basis, the unique stereoselectivity of AChE towards these types of CW agents.

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